Lipocortins in the central nervous system in multiple sclerosis and experimental allergic encephalomyelitis

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LIPOCORTINS IN THE CENTRAL NERVOUS SYSTEM IN MULTIPLE SCLEROSIS AND EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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SUMMARY

Corticosteroids are very effective in the treatment of acute relapse of multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS). Furthermore, steroids also have the ability to suppress an animal model of this condition, experimental allergic encephalomyelitis (EAE). It has been proposed that the anti-inflammatory effects of corticosteroids may be mediated, in part, via the induction of effector proteins termed lipocortins. This thesis has investigated whether the potential exists for lipocortins to play a role in the mechanism of action of steroids in MS and EAE.

Using Western blotting immunoreactive lipocortins I, II, IV and V were detected in normal human CNS tissues and lipocortins I, II and V in rat CNS samples. Increased levels of all lipocortins were found in diseased CNS tissues from MS patients and elevated amounts of lipocortin I in the CNS of rats with EAE.

Immunolocalisation of lipocortin I revealed that in normal CNS tissue the protein was present in the walls of blood vessels. Increased lipocortin I immunoreactivity in MS was found to be due to expression of the protein by activated astrocytes, and in EAE to result from widespread immunostaining of infiltrating lymphocytes and macrophages. Measurement of corticosterone in the sera of EAE-inoculated rats showed that changes in lipocortin I immunoreactivity in the CNS closely paralleled fluctuations in endogenous corticosteroid.

To determine whether the levels of lipocortins I, II and V in the CNS might be influenced by exogenous or endogenous corticosteroids, the effect of manipulating the circulatory steroid concentration during EAE was investigated. Dosing EAE-diseased rats with corticosterone or dexamethasone appeared to have no influence on the amount of lipocortin in the cervical spinal cord. Moreover,

administration of the glucocorticoid antagonist RU 38486 or adrenalectomy prior to inoculation did not prevent the disease-associated increase in lipocortin I immunoreactivity. These findings suggest that steroids do not modulate the absolute amount of lipocortin in the CNS.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Adx	Adrenalectomy/adrenalectomised
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BICINE	N,N-bis(2-hydroxyethyl)glycine
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CO_2	Carbon dioxide
COP 1	Copolymer 1
CPM	Counts per minute
CREAE	Chronic relapsing experimental allergic encephalomyelitis
CRF	Corticotrophin-releasing factor
CSF	Cerebrospinal fluid
DAB	3,3'-diaminobenzidine
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	[Ethylenebis(oxyethylenenitrilo)]tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
GFAP	Glial fibrillary acidic protein
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic-pituitary-adrenal
hr	Hour(s)
IFN	Interferon

IgG	Immunoglobulin G
IL	Interleukin
LT	Leukotriene
MBP	Myelin basic protein
MHC	Major histocompatability complex
min	Minute(s)
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MWM	Molecular mass markers
Ni/Co	Nickel/Cobalt
NSAIDs	Non-steroidal anti-inflammatory drugs
NSB	Non-specific binding
PAF	Platelet activating factor
PAP	Peroxidase-anti-peroxidase complex
PBS	Phosphate buffered saline
PG	Prostaglandin
PI	Post-inoculation
PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulphonyl fluoride
RIA	Radioimmunoassay
rh	Recombinant human
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sub. cut.	Subcutaneously
TEMED	NNN'N'-tetramethylethylenediamine
TCR	T cell receptor
TRIS	Tris(hydroxymethyl)aminoethane

INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) was first identified and characterised by Charcot as long ago as 1868. The name of the disease is descriptive and was derived from the characteristic multiple grey sclerotic lesions (or plaques) which were found at post-mortem in the brain and spinal cord of patients dying with MS.

Today MS is considered to be the leading cause of serious neurological disease in young and middle-aged adults in Western Europe and the United States (Johnson *et al.* 1979). In the United Kingdom between 50,000 and 100,000 people are affected by the disease. Although much has been learned about the disease since Charcot's time: in the last 15 years alone more than 10,000 articles about MS have been published (Poser 1992), at present the aetiology of MS is still unknown, the pathogenesis of the disease remains debatable and there is no definitive treatment.

1.1.1 Clinical Aspects

The first symptoms of MS can occur anytime between the ages of 15 and 50 but the maximum incidence is at 20-30 years of age. Although both men and women are susceptible, the disease it is more prevalent in females (Cuzner & Davison 1979).

One aspect of MS which hampers research on the disease is that the clinical course is extremely variable and unpredictable (McFarlin & McFarland 1982a). The majority of patients (approximately two thirds) are affected by a relapsing and remitting type of MS which is characterised by acute exacerbations of neurologic dysfunction followed by periods of remission. The average time to recovery is between 6 and 12 weeks and in the early stages remission may be complete with neurological function returning to normal. In the later stages there is less

improvement with each remission and the disease gradually enters a chronic phase of progressive neurological impairment.

Other less common disease types include progressive MS which is characterised by a continuous but gradual decline in neurologic function, and the rare acute form where deterioration is rapid and can be terminal within a few months. A benign type of MS also exists where one or a few mild exacerbations are followed by complete recovery. In addition, there is evidence that MS occurs subclinically, because MS-type lesions are occasionally found at postmortem in asymptomatic patients.

The symptoms of MS are also highly variable but tend to reflect the involvement of the white matter. The most common presenting symptoms are sensory and visual disturbances, and weakness or incoordination of the limbs (Swanson 1989). As the disease progresses patients may develop persistent problems with fatigue, spasticity, pain, tremor, bowel, bladder and sexual dysfunction, paroxysmal phenomena, depression and cognitive impairment (Noseworthy 1991). For most patients however, diagnosis of MS carries a reasonable prognosis: the majority are still ambulatory 25 years after onset (Percy *et al.* 1971) and the disease is rarely lethal.

1.1.2 Histology and Pathology

Lesions in the central nervous system (CNS) of patients with MS vary in size and may be up to several centimetres in diameter. The majority are found scattered throughout the white matter but there are distinct regions of predilection, the most commonly affected areas being the optic nerves, brain stem, spinal cord and the periventricular white matter of the cerebral hemispheres (Lumsden 1970). Although the symptoms of MS tend to reflect the involvement of particular areas of the CNS, the correlation between the number and distribution of plaques and

clinical symptoms is often quite poor (Thompson *et al.* 1990). Recent serial magnetic resonance imaging (MRI) studies have shown that despite the relapsing-remitting nature of the symptoms, the disease process is continually active: patients frequently exhibit many more new lesions than relapses and the lesions are continually changing in size, with some fading whilst others are simultaneously growing and becoming confluent (Koopmans *et al.* 1989).

Histologically lesions are generally classified as acute or chronic (see: Adams 1976, Weller 1985). Acute plaques are soft and oedematous and characterised by evidence of active myelin breakdown and widespread inflammation (Adams *et al.* 1989). Throughout the plaque, destruction of the myelin sheath is accompanied by some loss of oligodendrocytes although neurones are relatively well preserved. Demyelinated areas are hypercellular due to infiltration by inflammatory cells and proliferation of astrocytes. Monocytes and lymphocytes accumulate in the perivascular compartment between the walls of blood vessels and the glial basement membrane forming thick perivascular cuffs. Many of these cells migrate into the parenchyma and large numbers of lymphocytes, macrophages and plasma cells are found throughout the lesion. At the plaque margins, foamy macrophages containing myelin breakdown products are found closely associated with demyelinating axons. Glial hyperplasia occurs at the periphery with the accumulation of numerous reactive astrocytes and microglia in this area. There is also some increase in oligodendrocyte numbers and evidence of scanty remyelination at the plaque edge.

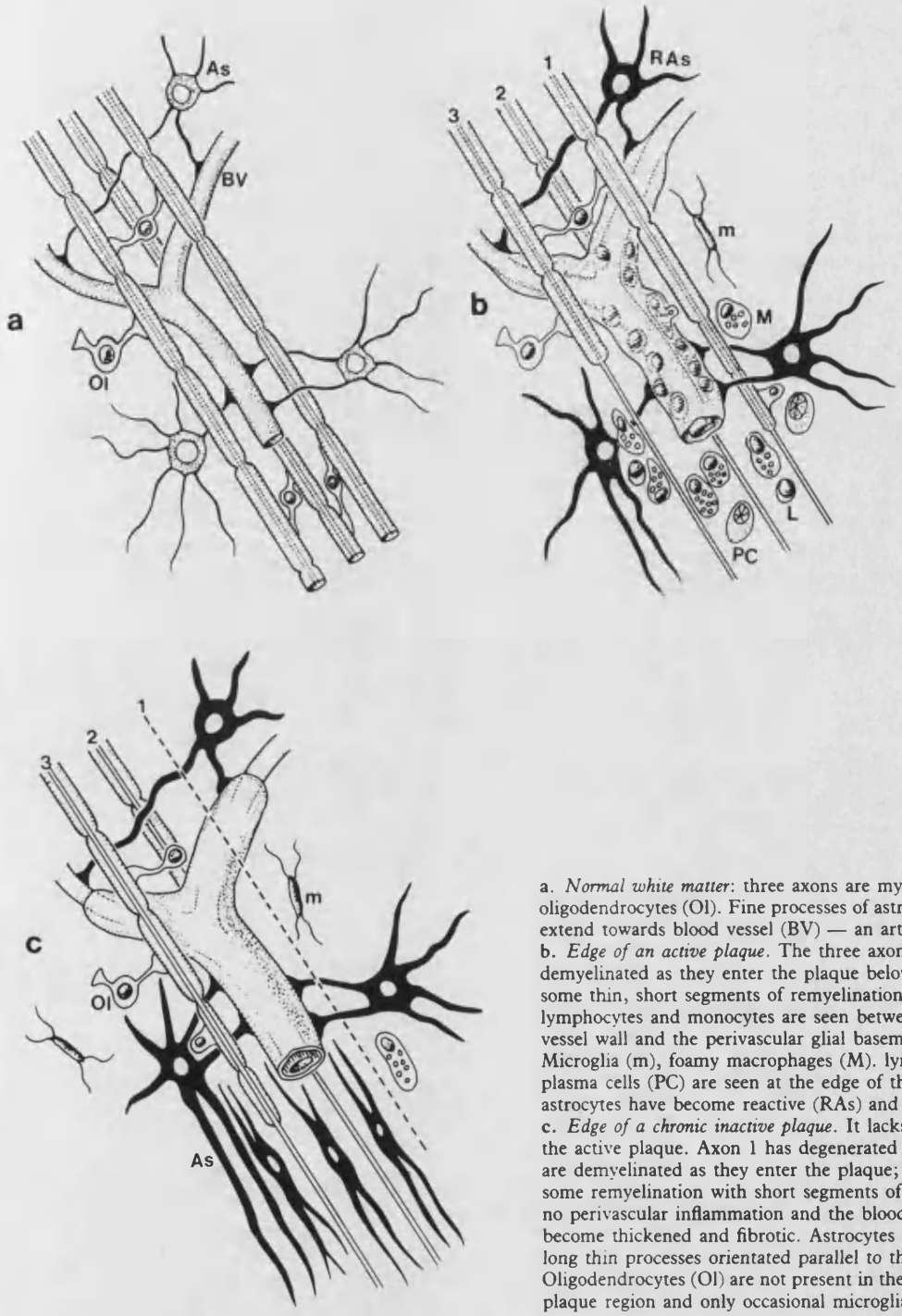
Chronic plaques are much more common and can be subdivided as either active or inactive. Chronic active plaques are persistently oedematous (McDonald & Barnes 1989) and exhibit evidence of continued demyelination and inflammation at the periphery. The core is demyelinated, hypocellular and gliotic, few oligodendrocytes remain and there is some degeneration of axons. Active myelin

breakdown is confined to the rim of the plaque which is hypercellular and contains many perivascular cuffs of lymphocytes and plasma cells together with large numbers of microglia, foamy macrophages and reactive astrocytes (Prineas & Wright 1978). As in acute plaques a slight increase in oligodendrocytes and a small amount of remyelination are found at the plaque edge.

Chronic inactive plaques lack the hypercellular rim of active plaques, show no evidence of further demyelination and appear to be "burnt out". They are of a rubbery consistency, hypocellular and densely gliotic. In the demyelinated area oligodendrocytes are no longer present and axonal loss may be substantial. The plaque is repaired by astrocyte fibres and processes from these cells are often observed surrounding preserved axons. Blood vessels frequently have thick collagenous walls which probably reflects the presence originally of perivascular cuffs. Oligodendrocytes are normal in number at the periphery and there is no further remyelination.

A pictorial summary of the different types of plaque is shown in Figure 1. Both active and inactive plaques may be found within the same brain; these histological variations appear to represent different ages of plaque and it seems probable that lesions initially develop as acute plaques, progress through a chronic active stage and then eventually become inactive (Adams 1976, Weller 1985).

What triggers the formation of the initial or subsequent lesions is not known. Plaques tend to develop around small cerebral blood vessels, usually veins. Recent MRI and histological studies have shown that breakdown of the blood-brain barrier (BBB) is a very early event which precedes cellular infiltration (McDonald & Barnes 1989, Kermode *et al.* 1990, Newcombe *et al.* 1991) and it has been proposed that these foci of BBB leakage may represent the primary lesion in MS (Miller *et al.* 1988).



a. *Normal white matter*: three axons are myelinated by oligodendrocytes (Ol). Fine processes of astrocytes (As) extend towards blood vessel (BV) — an artery or a vein.
b. *Edge of an active plaque*. The three axons are demyelinated as they enter the plaque below. Axon 1 shows some thin, short segments of remyelination. Perivascular lymphocytes and monocytes are seen between the blood vessel wall and the perivascular glial basement membrane. Microglia (m), foamy macrophages (M). lymphocytes (L) and plasma cells (PC) are seen at the edge of the plaque. The astrocytes have become reactive (RAs) and hypertrophied.
c. *Edge of a chronic inactive plaque*. It lacks the cellularity of the active plaque. Axon 1 has degenerated and axons 2 and 3 are demyelinated as they enter the plaque; axon 3 shows some remyelination with short segments of myelin. There is no perivascular inflammation and the blood vessel wall has become thickened and fibrotic. Astrocytes (As) have many long thin processes orientated parallel to the surviving axons. Oligodendrocytes (Ol) are not present in the demyelinated plaque region and only occasional microglia (m) are seen.

Figure 1. A diagrammatic summary of the histological features of multiple sclerosis

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The cause of the influx of cells from the blood into the CNS has not been identified although it has been suggested that expression of Class II major histocompatibility complex (MHC) antigens by endothelial cells may promote passage of activated T lymphocytes across the BBB (Traugott *et al.* 1985). Early inflammatory events appear to be T cell orchestrated (Raine & Scheinberg 1988) with acute/peripheral cuffs being comprised predominantly of lymphocytes (Cuzner & Davison 1979). Subsequent mediator release probably then causes recruitment of other inflammatory cells, and chronic-type cuffs within plaques, are composed mainly of plasma cells and monocytes (Jellinger 1977).

Ongoing demyelination appears to be dependent upon the presence of macrophages (Traugott *et al.* 1983), in early lesions these appear to be haematogenous in origin but in more established plaques, resident microglia also appear to become involved (Adams *et al.* 1989). Within areas of myelin loss foamy macrophages containing myelin debris are found in close contact with demyelinating sheaths (Prineas *et al.* 1984, Weller 1985) and in addition to the role played by these cells as lipid scavengers there is evidence that they may actively strip myelin from intact axons by phagocytosis (Prineas & Connell 1978). Soluble mediators involved in demyelination have not been identified but it has been proposed that macrophage lysosomal enzymes may be responsible for the initial attack on myelin (Einstein *et al.* 1972, Adams 1977). Selective loss of myelin basic protein (MBP) and increased proteolytic activity have been detected in MS plaques (Einstein *et al.* 1972, Bowen & Davison 1974). In addition, there is a marked loss of phospholipid in demyelinated tissues (Cuzner 1980) and the lipolytic enzyme phospholipase A_2 (PLA_2) has also been implicated in demyelination (Woelk *et al.* 1974,1976).

Established plaques are persistently oedematous and appear to enlarge through cycles of renewed peripheral activity (McDonald & Barnes 1989). With

time the centre of the plaque becomes inactive and eventually the whole lesion may become a gliotic, chronic inactive plaque. It has been suggested that the extensive gliosis which is a feature of inactive areas is probably a reaction to tissue damage rather than part of the mechanism of demyelination (Weller 1985).

A major discrepancy between the pathology of MS and the clinical course of the disease is the question of what forms the basis of remissions. Remyelination does not appear to be the cause, because although there is a limited amount of remyelination at the edge of many plaques, in most the majority of axons remain demyelinated (Prineas & Connell 1979). One proposal is that recovery is caused by restoration of conduction in demyelinated nerves, and it has been postulated that this may be due to proliferation of sodium channels along the axonal membrane, thus allowing conduction in the absence of myelin (Bostock & McDonald 1982), however there is little evidence for this mechanism. Another possibility which has received support from CNS imaging studies (Sears *et al.* 1978), is that the extensive oedema which pervades and surrounds the lesions may be responsible for temporarily inhibiting conduction in many neurones, either by increasing local pressure, causing delamination of myelin, or altering the ionic environment (Waksman & Reynolds 1984). Thus restoration of conduction and the rapid reversal of functional loss may simply be due to regression of oedema.

1.1.3 Aetiology

Familial studies suggest that susceptibility to MS is inherited: the incidence of MS being 15-20 times higher in first degree relatives than in the general population (McDonald & Halliday 1977). Concordance for MS in identical twins however, is relatively low (26%) (Ebers *et al.* 1986) indicating that although there is a genetic component in susceptibility, the pattern of inheritance is not simple.

In genetic studies MS has been found to be associated with particular MHC antigens. In Caucasians the Class I human leukocyte antigens A3 and B7 occur more commonly in patients with MS (Batchelor *et al.* 1978) and the disease is also linked to some Class II specificites such as DR2 which is present 3 to 4 times more frequently in MS patients than in the general population (Oger & Arnason 1980). MHC antigens appear to be involved in the control of immune regulation but the significance of these associations is not yet known.

Epidemiological research has shown that MS is not uniformly distributed throughout the world. There is a marked variation according to latitude, the prevalence being much greater in temperate (30-80 cases per 100,000 population) than in tropical or sub-tropical regions (<5 per 100,000) (Kurtzke 1980). Migration studies on people moving from high risk to low risk areas (and vice versa) indicate that migrants who relocate during childhood acquire the prevalence of their host area, but those migrating as young adults retain the prevalence of their place of origin (Alter *et al.* 1978), suggesting that susceptible individuals acquire the disease from an environmental factor during adolescence.

Mainly as a result of studies on the epidemiology and pathology of the disease, two opposing theories have developed as to the cause of MS: one that MS is due to an infectious agent and the other that it is an autoimmune disease.

The idea that MS is the result of a slow infection by a virus or some other organism originates primarily from epidemiological studies. The main points of evidence for this hypothesis are: the similarity between the lesions of MS and those observed in viral diseases such as progressive multifocal leukoencephalopathy and subacute sclerosing panencephalitis caused by measles virus (Graves 1984); the presence of increased immunoglobulin G (IgG) and oligoclonal bands in the cerebrospinal fluid (CSF) of MS patients (Walsh *et al.* 1983) which is a feature of many other infectious diseases where the antibody is directed against the aetiologic

agent; the detection of antibody to measles (Adams & Imagawa 1962) and other viruses (Norrby *et al.* 1974) in the CSF and serum of patients with MS; and evidence from animal models that viral infection can cause demyelinating lesions similar to those of MS (Rodriguez 1989). Although many candidates have been proposed as the causative agent of MS including spirochetes, mycoplasma, protozoa and several viruses (see: McFarlin & McFarland 1982b), the strongest argument against this theory is that no infectious organism has been consistently isolated from the brain or other tissues of patients with MS. Furthermore, it has not been possible to transmit the disease to primates by inoculation with infected CNS tissue (Sibley *et al.* 1980).

It was first suggested that MS might be immunologically mediated due to the presence of inflammation and tissue damage occurring in the absence of a known infectious agent and the observation of similarities in the pathology of MS, other human autoimmune diseases and encephalomyelitis induced in animals by administration of CNS tissue (Waksman & Reynolds 1984). There is considerable evidence of an active immune response in the CNS of MS patients. Intrathecal production of immunoglobulins occurs in 90% of patients and antibodies to MBP and other CNS components have been detected in the CSF (Walsh *et al.* 1983), findings which suggest involvement of the humoral immune system. The presence of large numbers of T lymphocytes and macrophages in MS lesions (Prineas & Wright 1978) indicates that cell-mediated immunity may be important in disease pathogenesis. In addition, various immune abnormalities in the peripheral blood of patients with MS point to immune regulatory changes in the disease. Furthermore, the observation that some immunosuppressive therapies have beneficial effects also suggests that immune mechanisms are important in MS (Waksman & Reynolds 1984).

Many workers in the field now support the theory that both infection and autoimmunity play a role in the pathology of MS. Several hypotheses have been put forward (see: McKhann 1982, McFarlin & McFarland 1982b, Rodriguez 1989) suggesting that infection of either the immune system, oligodendroglia or other CNS cells, triggers directly or indirectly, an autoimmune reaction to components of the CNS which results in demyelination. At present none of these hypotheses has been proven or disproven and whether the process begins in the CNS or at the peripheral level is not clear.

1.1.4 Treatment of MS

The cause and pathogenesis of MS are unknown and at present no preventative measures or definitive therapies exist. Treatments used in MS can be divided into three groups: 1) those used to control individual symptoms (which are not discussed here), 2) experimental therapies aimed at altering the course of the disease and 3) adrenocorticotrophic hormone (ACTH) and corticosteroids which are used clinically to treat acute relapses. Unfortunately the unpredictability of the disease, the occurrence of spontaneous remissions, a marked placebo influence and the absence of any reliable indicators of disease activity make it very difficult to effectively evaluate potential therapies for MS.

As the pathology of MS appears to have an immunologic component there is a rationale for treatment with immunosuppressive therapy. Since the early 1960's numerous immunosuppressive and immunomodulatory agents have been tried in MS, the most extensively studied being the cytostatic global immunosuppressives, cyclophosphamide and azathioprine.

Cyclophosphamide is an alkylating agent which acts mainly by cross-linking DNA. It has been used in Europe for more than 20 years and several early uncontrolled studies concluded that cyclophosphamide reduced relapse rate and

slowed disease progression (Kappos 1988). These conclusions were confirmed by two controlled clinical trials (Hauser *et al.* 1983, Goodkin *et al.* 1987) but because of side effects such as nausea, vomiting, alopecia, bladder toxicity and possible induction of malignancies, the drug was recommended only for patients with rapidly progressive disease. Recently lower doses of cyclophosphamide have been reported to be effective in both progressive (Mauch *et al.* 1989) and relapsing-remitting MS (Killian *et al.* 1988) with only minor side effects, however other workers have been unable to find a beneficial effect (Likosky 1988) and the drug is not recommended as a routine treatment.

Azathioprine, a phase-specific anti-metabolite, has been more extensively used in MS than any other immunosuppressive agent, partly due to its relatively low toxicity. In early uncontrolled studies (Kappos 1988) azathioprine was found to be without effect in chronic progressive MS but was reported to reduce relapse rate and disease progression in the majority of patients with relapsing-remitting disease. However in a large multi-centred, double-blinded placebo-controlled study by the British and Dutch Multiple Sclerosis Azathioprine Trial Group (1988) the drug was found to be no better than placebo in the first two years, to have only marginal effects on disease progression in the third year and it was concluded that the benefits obtained were too small to justify the use of azathioprine in relapsing and remitting MS.

Cyclosporin A is a newer immunomodulatory agent which has a specific and reversible effect on T lymphocyte function and is believed to act primarily by preventing the production of interleukin-2 (IL-2). In a clinical trial comparing low dose cyclosporin A with azathioprine it was found that although there was no significant difference with respect to clinical effect, there was a much higher incidence of side effects such as nephrotoxicity and elevated blood pressure in cyclosporin treated patients (Kappos *et al.* 1988). Furthermore, following a recent

double-blind trial of two doses of cyclosporin A versus placebo, the authors concluded that the side effects were of such severity as to preclude the use of cyclosporin in a high enough dose to alter the course of the disease (Rudge *et al.* 1989).

The anti-viral and immunomodulatory properties of the interferons (IFNs) have lead to clinical trials of IFNs α, β and γ in MS. Several uncontrolled studies on the use of IFN-α showed a slight therapeutic benefit, but in a large placebo controlled trial IFN-α did not significantly alter disease progression and was associated with a substantial number of adverse drug reactions (AUSTIMS 1989). Intrathecally administered IFN-β has been found to be effective in reducing exacerbations in one controlled trial, however there was no significant effect on disease progression and the route of administration is potentially hazardous (Jacobs *et al.* 1987). In one pilot study of IFN-γ given to 18 patients with relapsing-remitting MS, 7 out of 18 developed an acute exacerbation within the first month (Panitch *et al.* 1987). IFN-γ is an immune enhancer and other immune activating drugs such as levamisole (Dau *et al.* 1976) and isoprinosine (Confavreux *et al.* 1986) have also been shown to increase relapse rate and accelerate disease progression.

Physical non-pharmacological methods of immunosuppression such as plasma exchange (Tindall 1988) and total lymphoid irradiation (Cook *et al.* 1987) have also been tried in MS, however conflicting reports exist and the efficacy of these procedures has not been proven.

In general the beneficial effects of global immunosuppression in MS appear to be relatively small, transient and associated with significant toxicity. Recently more disease-specific approaches have been assessed as potential therapies for MS. Desensitisation with MBP proved ineffective (Campbell *et al.* 1973) however a synthetic peptide, Copolymer 1 (COP 1) originally designed as an MBP analogue

appeared to cause some reduction in relapse frequency and disease progression in patients with early exacerbating and remitting MS, unaccompanied by serious side effects (Bornstein *et al.* 1987). COP 1 is non-toxic and further trials are underway; its mechanism of action in MS is not known.

Many different therapies have been tried in MS (see: Arnason *et al.* 1982) including other immunosuppressive/immunomodulatory compounds and procedures such as d-penicillamine, cytosine arabinoside, selective histamine and serotonin antagonists, transfer factor, thymic hormones, immunoglobulins, anti-lymphocyte serum, lymphocytapheresis, thoracic duct drainage, thymectomy and combined treatments; anti-inflammatory agents such as chloroquine and the non-steroidal anti-inflammatory drugs (NSAIDs); dietary supplements, particularly those rich in polyunsaturated fatty acids; and other miscellaneous treatments for example protease inhibitors, superoxide dismutase, calcium chelating agents, calcium channel blockers, potassium channel blockers and hyperbaric oxygen. Some have been shown to be ineffective, others are still under investigation, many are toxic, and in common with the treatments described earlier none is recommended for routine clinical use.

1.1.5 Corticosteroid Therapy in MS

Corticotrophin and corticosteroids were first investigated as possible treatments for MS because of their ability to suppress allergic and inflammatory reactions, their successful use in the therapy of various autoimmune disorders, and because they were effective in suppressing animal models of the disease. Early clinical trials of ACTH and steroids in MS produced conflicting results (Merritt *et al.* 1954, Miller *et al.* 1961ab, Tourtellotte & Haerer 1965). However in 1970 the National Co-operative ACTH Study Group (Rose *et al.* 1970) published the results of a large multi-centred double-blind study comparing corticotrophin therapy with

placebo in 197 patients in acute exacerbation, which demonstrated that ACTH hastened the improvement of signs and symptoms and that the treatment was associated with no major complications or side effects.

In the 20 years since this report was published the use of ACTH and corticosteroids for the treatment of acute exacerbations of relapsing-remitting MS has become common in neurologic practice (Troiano *et al.* 1987). There is no consensus as to whether ACTH or corticosteroids are superior in the treatment of MS (Davis & Stefoski 1988, Trioano *et al.* 1989, Poser 1989). Both compounds accelerate recovery from relapse thus reducing the duration of disabling symptoms, however there is no evidence that they enhance the degree of recovery or modify the course of the disease. Side effects associated with short courses of treatment are mild and relatively infrequent, the most common reported by Rose *et al.* (1970) being: acne (31%), moon face (7%), hirsutism (7%), impaired glucose tolerance (5%) and weight gain (5%).

Recently much attention has been focused on the use of pulsed, high-dose, intravenous steroids which in early uncontrolled studies showed a very rapid onset of improvement, occurring in some cases overnight, or even during perfusion (Buckley *et al.* 1982, Goas *et al.* 1983). Subsequent controlled trials confirmed that high dose intravenous methylprednisolone was significantly better than placebo in both chronic progressive and relapsing-remitting MS (Durelli *et al.* 1986, Milligan *et al.* 1987) although in comparative studies this form of treatment appeared to be, at best, only marginally more effective than ACTH (Abbruzzese *et al.* 1983, Barnes *et al.* 1985, Thompson *et al.* 1989). Nevertheless, high dose intravenous steroids are becoming more widely used. The main advantages are that they are more easily administered, have a more rapid onset of action (typically 3-6 days) and are free of serious side effects due to the short duration of use (Lyons *et al.* 1988).

Many early studies demonstrated that chronic treatment of MS with conventional doses of steroids had no long term benefit on disease progression in either relapsing-remitting or chronic progressive MS (Merritt *et al.* 1954, Miller *et al.* 1961a, Fog 1965, Tourtellotte & Haerer 1965, Miller *et al.* 1967). Recently it has been suggested that long-term high dose corticosteroids (Troiano *et al.* 1987) or repeated pulses given at regular intervals (Polman *et al.* 1991) might be effective in preventing disease progression. Unfortunately, with this type of regimen, there is a risk that patients may develop serious complications such as osteoporosis, muscle wasting, recurrent infections, cataracts and psychosis.

The mechanism by which ACTH and corticosteroids hasten recovery from acute relapse of MS is not known. It is probable that ACTH acts indirectly by stimulating release of endogenous glucocorticoids from the adrenal cortex, although it has been postulated that corticotrophin may have direct effects upon the immune system and neural tissue independent of corticosteroid release (Davis & Stefoski 1988, Poser 1989). Three main theories exist as to the mode of action of steroids in MS: 1) a direct effect on nerve conduction, 2) an immunologically mediated mechanism and 3) resolution of oedema.

Although there is some experimental evidence that steroids may directly influence conduction along demyelinated axons (Arnason & Chelmicka-Schorr 1974) which, it has been suggested, may be due to changes in brain electrolyte concentrations (Troiano *et al.* 1987), there is little evidence for this mechanism in MS.

Steroids have numerous effects on the immune system and many studies have reported normalising effects of steroid treatment on immunologic abnormalities present in MS. Elevated intrathecal IgG synthesis and oligoclonal band intensity are substantially decreased following treatment with ACTH and corticosteroids (Tourtellotte *et al.* 1980, Durelli *et al.* 1986). Furthermore,

abnormally high CSF leukocyte numbers (Naess & Nyland 1981), free MBP and antibody to MBP (Warren *et al.* 1986) plus immune complexes and antibody to myelin associated glycoprotein (Wajgt *et al.* 1983) have all been shown to be reduced by steroid therapy. Peripherally, steroid treatment has been found to return elevated levels of serum soluble IL-2 receptor to normal (Bansil *et al.* 1991) and to reduce the number of circulating T lymphocytes (Kaschka & Hilgers 1980). It should be noted however that other groups have found no significant effects of steroid treatment on immunologic abnormalities in MS (Compston *et al.* 1987). Moreover the importance of these parameters in the pathology of exacerbations is not known.

Recently evidence has accumulated from studies using gadolinium enhanced MRI and computed tomographic scanning which suggests that blockade of nerve conduction in MS is caused by local tissue inflammation and oedema, and that the main mechanism of steroid action may be to reverse these processes. Several studies have shown that high dose intravenous methylprednisolone substantially decreases both the signal intensity and the apparent number of active lesions, indicating a reduction in vascular permeability and resolution of oedema (Sears *et al.* 1978, Trioano *et al.* 1984,1987, Barkhof *et al.* 1991, Burnham *et al.* 1991). As with clinical improvement, the onset of this effect is very rapid often occurring within 8 hours of treatment (Troiano *et al.* 1984). Presumably restoration of BBB integrity also restricts the passage of serum factors and immunocompetent cells from the blood into the CNS (Troiano *et al.* 1984) which may explain the reduction in inflammatory infiltrates observed in the brains of steroid treated MS patients at postmortem (Guseo & Jellinger 1975).

1.2 EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

1.2.1 Induction and Symptoms

Experimental allergic encephalomyelitis (EAE) is a paralytic autoimmune disease which is readily inducible in laboratory animals and is widely used as a model for MS. EAE was discovered early in the 20th century as a result of attempts to explain the occurrence of "paralytic accidents" in some patients receiving the Pasteur rabies vaccine. This vaccine consisted of fixed rabies virus prepared in rabbit CNS tissue and it was hypothesised by Remlinger (1905) that the "paralytic accidents" were caused by the nervous tissue rather than the viral component of the vaccine. Subsequently it was shown that rabbits (Stuart & Krikorian 1928) and monkeys (Rivers & Schwentker 1935) injected repeatedly with CNS tissue developed an acute disseminated encephalomyelitis which shared many of the clinical and histological features of post-rabies vaccine encephalitis, including the presence of mononuclear perivascular infiltrates and focal areas of demyelination in the CNS. These studies represented the first work on what is now known as EAE.

In 1947 a further advance was made when Freund and co-workers (Freund *et al.* 1947) discovered that inclusion of an adjuvant comprising killed bacteria, paraffin oil and an emulsifying agent with the CNS tissue inoculum, resulted in an accelerated form of the disease which was very reproducible and could be induced in several species, within 2-3 weeks, following only a single injection of CNS-adjuvant emulsion. Gradually it was realised that EAE had many histological and pathological similarities to MS and today EAE in its various forms is widely used as an experimental model for this disease.

The most extensively studied type of EAE is the acute form caused by active sensitisation to CNS antigen. This can be induced in a large number of

mammalian species including monkeys, rabbits, guinea pigs, rats and mice and also in some birds. Susceptibility varies considerably between strains: whereas Lewis rats are highly susceptible to EAE, many others such as the Brown Norway are strongly resistant to the disease (Levine & Wenk 1963). Active EAE is usually induced by subcutaneous depot injection in the flanks, nuchal region or foot pads, with an emulsion comprising brain or spinal cord homogenised 1:1 in saline or water plus an equal volume of complete Freund's adjuvant (CFA), a mineral oil containing an emulsifier and killed mycobacterium (Raine 1984). In the early 1960's the encephalitogenic, EAE inducing, activity of CNS tissue was found to be mainly due to MBP, the major protein constituent of myelin (Kies 1965). Indeed purified MBP is now frequently substituted for CNS tissue for the induction of EAE.

Following inoculation most animals develop an acute monophasic paralytic disease which is invariably fatal in the case of monkeys, rabbits, guinea pigs and mice although in the rat EAE is self-limiting and most animals recover. Rats sensitised for acute EAE usually develop the disease within 11-14 days of immunisation. Initially a sudden and dramatic weight loss is observed, followed over a period of about 48 hours by the development of an ascending flaccid paralysis starting with limpness of the tail, progressing through ataxic gait caused by weakness of the hind legs, to paresis or paralysis of both hind limbs, often accompanied by urinary incontinence. Although the symptoms may be very severe, the majority of rats recover completely within 4-5 days (Paterson 1976).

A chronic relapsing form of EAE (CREAE) can be induced in some animals. Juvenile strain 13 guinea pigs sensitised with spinal cord-CFA develop a milder chronic type of EAE, that relapses and remits throughout the life of the animal (Snyder *et al.* 1975). A similar disease is inducible in Lewis rats by

treatment with low doses of cyclosporin A (Polman *et al.* 1988) or cyclophosphamide (Minagawa *et al.* 1987).

In addition to active induction of the disease it is also possible to passively transfer EAE by injection of lymphoid cells taken from animals previously sensitised to CNS tissue (Paterson 1960). Following inoculation with donor lymphoid cells the recipients develop, about 2-6 days later, a clinical syndrome with CNS lesions which appear to be identical to those found in actively induced EAE. Stimulation of the cells *in vitro* with MBP greatly increases the efficiency of adoptive transfer (Driscoll *et al.* 1979).

1.2.2 Histology, Pathology and Comparison with MS

Animals inoculated for EAE develop lesions in the CNS consisting of focal areas of perivascular inflammation accompanied by varying degrees of myelin damage, which share many similarities with those found in the brains of patients with MS. In EAE as in MS, the white matter is more affected than the grey and lesions are most often found near the ventricular system, in the subcortical white matter and in the brain stem and spinal cord (Paterson 1978).

Lesions generally form around small veins or venules. The earliest abnormal findings observed in the CNS of EAE-inoculated animals are: increased permeability of the BBB (Oldstone & Dixon 1968); expression of Ia antigens by CNS vascular endothelium (Hickey *et al.* 1985); and a low grade diffuse infiltration of the CNS by T lymphocytes (Traugott *et al.* 1982). These changes occur within the first few days of inoculation, well before the appearance of neurological signs (Juhler 1988, Traugott 1989).

Just preceding the onset of symptoms, extensive vasogenic oedema develops in the CNS (Leibowitz & Kennedy 1972, Oldendorf & Towner 1974) and there is a massive invasion of the target tissue by large numbers of inflammatory

cells (Paterson 1976, Raine 1984). Mononuclear cells cross the blood vessel walls and accumulate in the perivascular space forming thick concentric layers. These perivascular infiltrates are composed predominantly of monocytes/macrophages plus some B lymphocytes and a few plasma cells; T lymphocytes are found scattered throughout the white matter parenchyma (Traugott *et al.* 1982). Myelin breakdown occurs shortly after the arrival of mononuclear cells (Raine 1984). In acute EAE demyelination is slight, but in chronic-relapsing models myelin loss may be extensive (Shaw & Alvord 1984) and some remyelination occurs at the edge of lesions (Raine 1984). At the same time as the appearance of the first inflammatory infiltrates astrocytes proliferate and become hypertrophied (Smith & Eng 1988), and microglia accumulate particularly in the vicinity of the lesions (Matsumoto *et al.* 1986). In chronic EAE as the disease progresses, fibrous astrocytes move into demyelinated areas and form dense gliotic scars similar to those observed in MS (Raine 1984, Smith & Eng 1988).

In early studies it was observed that the lesions of EAE resembled those of delayed-type hypersensitivity reactions (see: Paterson 1976). Since that time, compelling evidence has accumulated that EAE is primarily a T cell-mediated disease, although B cell-mediated humoural mechanisms probably also play a role in the pathology (Waksman & Reynolds 1984, Juhler 1988). A strong indication that the disease had an immunological basis was provided by the observation that removal of lymph nodes draining the site of inoculation prevented the development of EAE (Condie & Good 1959) and this was substantiated by the demonstration that EAE could be passively transferred with sensitised lymphoid cells (Paterson 1960). The importance of thymic function was established by studies which showed that thymectomized rats were resistant to EAE (Arnason *et al.* 1962), but in the chicken, removal of the bursa of fabricius (the B cell organ in birds) did not significantly inhibit disease induction (Blaw *et al.* 1967). Later, complex

experiments in Lewis rats involving depletion of immune cells and reconstitution of the bone marrow with various lymphocyte subsets confirmed that T cells are a prerequisite for the induction of EAE (Gonatas & Howard 1974).

T lymphocytes are the first cells to appear in the CNS following inoculation. How these cells gain entry into what is normally an immunologically privileged site is not known, but it has been proposed that expression of Ia antigens by vascular endothelial cells may promote adherence and subsequent penetration of activated lymphocytes (Wisniewski *et al.* 1982), and increased BBB permeability may also aid entry of immune reactive agents normally excluded (Juhler 1988). Primary invasion of the CNS by a small number of specific T cells is then followed by recruitment of large numbers of activated non-specific monocytes/macrophages and smaller numbers of specific and non-specific T and B lymphocytes (Waksman & Reynolds 1984).

Demyelination is almost certainly attributable to macrophages. Electron microscopical studies indicate that there appear to be two different mechanisms by which these cells damage the myelin sheath: de-lamination via extension of cell processes between the myelin lamellae, and lysis caused by release of soluble factors (Lampert & Carpenter 1965, Wisniewski 1977). Myelinolysis is probably enzymatic and may be mediated by proteases (Cammer *et al.* 1978) and/or lipolytic enzymes (Vogel 1950) such as PLA_2, which is abundant in macrophages and has been found to digest myelin *in vitro* (Trotter & Smith 1984).

Although some workers have postulated that the clinical signs of EAE are due to demyelination and their disappearance a consequence of remyelination (Pender 1989), it has been argued that the transience of symptoms and the speed with which animals recover is not compatible with this mechanism (Simmons *et al.* 1981). Paterson (1982) found no relationship between neurological signs and the presence of inflammatory demyelinating lesions but did observe a close correlation

between signs and intra-CNS fibrin deposition, a phenomenon associated with accumulation of fluid in the extracellular space. Other studies have also demonstrated the occurrence of a dramatic increase in CNS vascular permeability concomitant with the onset of clinical symptoms (Leibowitz & Kennedy 1972, Oldendorf & Towner 1974, Simmons *et al.* 1982), and it has been speculated that build up of fluid within the physically limited space of the CNS compartment may cause increased pressure on nerve fibres, resulting in nerve dysfunction and the characteristic neurological signs of EAE (Paterson 1982, Simmons *et al.* 1982).

From the foregoing description of the clinical signs, histology and pathology of EAE, it is clear that although there are some important differences, the disease has many similarities with the human condition MS (see Table I for summary). It has been suggested that the course of disease and pathological changes in acute EAE are more analogous to early neuropathological events in MS, whereas the chronic relapsing model is more representative of the situation in chronic MS (Raine 1984) and moreover, where the human and animal diseases do diverge this is probably due to a difference in time scale rather than the nature of the disease process (Liebowitz 1983).

1.2.3 Modulation by Drugs and Immunotherapy

In the search for new drugs which might be of use in the treatment of MS, many compounds have been studied for their ability to influence the course of EAE. Most investigations have concentrated on inhibition/prevention (drug administered prior to sensitisation) or suppression (drug administered after sensitisation but before the onset of neurological signs). Fewer, because of the practical difficulties involved, have explored treatment of the disease, where the drug is administered therapeutically after signs have developed, although this is clearly most relevant to the clinical situation in MS. Despite the very large number

Table I

Summary of the main similarities and differences between MS and EAE

	EAE	MS
Relapsing and chronic paralysis	+	+
Predilection of lesions for white matter	+	+
Perivascular inflammatory infiltrates:	+	+
CD4+ T-cells	+	+
Macrophages	+	+
CNS demyelination	±	+
Axonal survival	+	±
Meningitis	+	+
Elevated CSF IgG	+	+
Antibodies to CNS antigens in CSF	+	+
IgG deposition in areas of injury	±	+
Circulating glial cell and myelinotoxic antibodies	+	+
Cutaneous DTH reaction to CNS antigens	+	0
Autoantigens	MBP, PLP	?
Linkage to MHC Class II	+	+
T-cell mediated pathogenesis	+	?
Known aetiology	+	-

Sources: Paterson 1978, Zamvil & Steinman 1990
DTH, delayed-type hypersensitivity; PLP, proteolipid protein

of drugs tested, the majority of active compounds fall into one of two categories: immunosuppressive/immunomodulatory agents and anti-inflammatory drugs.

Many of the early cytotoxic immunosuppressives were found to be effective in modulating EAE. In 1952 Kolb and co-workers reported that nitrogen mustard caused a significant decrease in paralysis and mortality of EAE-inoculated guinea pigs. Subsequently, other alkylating agents were shown to be active. Cyclophosphamide was found to completely prevent both the development of disease symptoms and the appearance of inflammatory lesions in the CNS of Lewis rats (Rosenthale *et al.* 1969). Furthermore, the drug reversed disease progression in rats with advanced paralytic signs (Paterson & Drobish 1969). Purine and pyrimidine analogues such as 6-mercaptopurine, thioguanine and azathioprine were also observed to be potent in preventing and suppressing the disease (Rosenthale *et al.* 1969, Komarek & Dietrich 1971), as was the folic acid antagonist methotrexate (Rosenthale *et al.* 1969).

Many of the newer immunosuppressive and immunomodulatory agents also exhibit disease modifying activity in EAE. Cyclosporin A, a fungal peptide with powerful immunosuppressive properties, inhibits both humoural and cell-mediated allergic reactions, probably by preventing the action and production of IL-2 (Borel *et al.* 1976). Prophylactic administration of cyclosporin A has been shown to prevent the appearance of clinical and pathological signs of EAE in rats, guinea pigs and primates (Bolton *et al.* 1982b). Furthermore in each species, treatment of established disease was found to reduce both the frequency and severity of symptoms and the number of CNS lesions. Cyclosporin A also inhibits adoptively transferred EAE if spleen cells are cultured with the drug prior to transfer, and the incidence of disease is reduced when recipient animals are pretreated with this agent (Bolton *et al.* 1982a). Unfortunately as discussed earlier, cyclosporin A appears to be too toxic for use in MS (Rudge *et al.* 1989).

FK506 is a relatively new immunomodulatory agent isolated from the fermentation broth of *Streptomyces tsukubaensis*. This compound probably has a similar mode of action to cyclosporin A although it appears to be more potent (Kino *et al.* 1987ab). FK506 has been shown to prevent the development of neurological signs of EAE in Lewis rats dosed during the induction phase of the disease. Also the cutaneous delayed-type hypersensitivity reaction to MBP and serum anti-MBP antibodies were completely suppressed in these animals (Inamura *et al.* 1988). Recently FK506 has been demonstrated to inhibit adoptive transfer of EAE (Bolton 1992). At present FK506 is undergoing clinical trials for use in the management of organ transplantation and has yet to be examined in MS.

Another immunosuppressive drug that appears to be active in EAE is 15-Deoxyspergualin, which has recently been shown to suppress acute EAE and to prevent relapses of CREAE in the Lewis rat (Schorlemmer & Seiler 1991). Again this compound has been used in organ transplantation, but has not yet been tested in MS.

It should be noted that not all immunomodulatory compounds found to be effective in EAE are of benefit in the treatment of MS. A case in point is IFN-γ, which has been shown to completely suppress clinical signs of EAE in the Lewis rat (Voorthuis *et al.* 1990) but cause an increase in the exacerbation rate when administered to patients with MS (Panitch *et al.* 1987).

The other main group of compounds containing many members effective in modulating EAE are the anti-inflammatory agents, which tend to act by inhibiting the generation of phospholipid derived pro-inflammatory mediators. Anti-inflammatory steroids which inhibit the production of both eicosanoids and platelet activating factor (PAF) are very effective in modulating EAE and will be discussed in detail in the next section. In contrast NSAIDs such as aspirin, indomethacin and phenylbutazone which prevent production of prostaglandins (PGs) by inhibiting

cyclo-oxygenase are ineffective (Rosenthale *et al.* 1969) and may even exacerbate the disease (Bolton & Cuzner 1980, Ovadia & Paterson 1982), although it is not known whether this is caused by specific immunological enhancement related to inhibition of PG synthesis or due to increased toxicity resulting from disease related metabolic disturbances (Levine & Sowinski 1980). Interestingly, the dual cyclo-oxygenase/lipoxygenase inhibitor BW755C which blocks generation of both PGs and leukotrienes (LTs) has recently been shown by Prosiegel *et al.* (1989a) to suppress EAE in guinea pigs but unfortunately this compound is too toxic for use in humans. The same group have demonstrated that sulphasalazine, which has a selective inhibitory effect on LTs, is also effective and they have suggested that the drug, which is frequently used clinically in the treatment of inflammatory bowel disease, be investigated for activity in MS (Proseigel *et al.* 1989b). However, it should be noted that other workers have reported sulphasalazine to have no beneficial effects in EAE (Uitdehaag *et al.* 1991, Correale *et al.* 1991). Other mechanistically related compounds that have recently been tested in EAE are the LTB_4 receptor antagonist SC-14930 which was found to cause some inhibition of EAE in the guinea pig (Fretland *et al.* 1991) and the PAF antagonists PCA 4248 and WEB 2170 which do not appear to protect against the development of EAE in the Lewis rat (Vela *et al.* 1991).

Another approach to the treatment of MS has been to investigate ways of specifically manipulating the immune system. It has long been known that EAE can be prevented, suppressed and treated by administration of whole white matter, purified MBP, MBP fragments and random copolymers such as COP 1 (see: Paterson 1976,1978). This early work has led to more sophisticated approaches and one strategy which is currently being pursued is prevention of T cell activation by inhibition of the interaction between antigen, MHC and the T cell receptor (TCR) using either MBP peptides (Sakai *et al.* 1989), anti-class II MHC antibodies

(Steinman *et al.* 1981) or anti-TCR antibodies (Owhashi & Heber-Katz 1988). Another approach which is being investigated is vaccination against autoreactive T cells by immunisation with attenuated T lymphocytes (Ben-Nun *et al.* 1981) or synthetic T cell receptor peptides (Vandenbark *et al.* 1989, Howell *et al.* 1989). With the exception of MBP, which was found to be ineffective (Campbell *et al.* 1973), and COP 1, which appeared to be of only limited benefit (Bornstein *et al.* 1987), specific immunotherapy has yet to be tried in MS.

1.2.4 Exogenous and Endogenous Steroids in EAE

A large number of studies have shown that prophylactic dosing with corticosteroids or corticotrophin beginning at or just after sensitisation, is very effective in suppressing both the neurological signs and CNS lesions of EAE in a variety of species including rats (Rosenthale *et al.* 1969, Komarek & Dietrich 1971, Bolton & Flower 1989), guinea pigs (Moyer *et al.* 1950, Field & Miller 1962) and monkeys (Kabat *et al.* 1952). In a comparative study in the rat, 19 out of 21 different steroid compounds were observed to be active in preventing EAE and fluorinated steroids were found to be considerably more potent than either alkylating agents or anti-metabolites (Komarek & Dietrich 1971). Corticosteroids are also effective therapeutically. In rodents administration of steroids after the onset of neurological signs reduces the severity of symptoms and shortens disease duration, indicating that steroids have the ability to reverse established disease (Greig *et al.* 1970, Levine & Sowinski 1980, Steiner *et al.* 1991). Furthermore, it has been demonstrated that passive transfer of EAE may be inhibited either by dosing the recipient animals with dexamethasone (Levine & Strebel 1969), or by incubation of sensitised cells with physiological concentrations of corticosterone prior to transfer (Leonard *et al.* 1990).

Recently a great deal of evidence has accumulated that endogenous corticosteroids may influence disease activity in EAE. Environmental stress such as that caused by enforced physical restraint or electric shock, applied after immunisation, has been shown to suppress the development of clinical and histological signs of both actively induced (Levine *et al.* 1962a, Bukilica *et al.* 1991) and adoptively transferred EAE (Levine & Strebel 1969), an effect which is probably mediated by stress-induced release of corticosteroids from the adrenal cortex. Moreover, it appears that endogenous adrenal steroids are responsible for the spontaneous recovery from EAE which is observed in some species. Several studies in the rat have demonstrated that plasma corticosterone levels rise dramatically with the onset of neurological symptoms, peak just before the initiation of recovery and then return to basal levels following remission (Levine *et al.* 1980, MacPhee *et al.* 1989, Mackenzie *et al.* 1989, Leonard *et al.* 1990). If this glucocorticoid surge is prevented by removal of the adrenals prior to inoculation (Levine *et al.* 1962b, MacPhee *et al.* 1989) or antagonised by treatment during induction with the anti-glucocorticoid RU 38486 (Bolton & Flower 1989) disease severity is markedly increased and recovery usually abolished. The effect of adrenalectomy (Adx) on disease progression can be completely reversed by corticosteroid replacement therapy at levels which mimic those normally produced during EAE (MacPhee *et al.* 1989). Interestingly Adx (Levine *et al.* 1980) or anti-glucocorticoid treatment (Bolton & Flower 1989) of convalescent rats can precipitate a relapse of symptoms indicating that endogenous steroids may be important not only for the induction, but also the maintenance of clinical remission.

The mechanism of action of exogenous and endogenous steroids in recovery from EAE has not been explored in detail. Glucocorticoids have powerful anti-inflammatory effects which appear to be due to their inhibitory action on inflammatory mediator production (Flower 1988), but they also suppress the

immune response in a number of ways (Fauci 1979) and influence the permeability of the blood-brain barrier (Long & Holaday 1985). Presumably any or all of these effects may be relevant to their mode of action in EAE.

1.3 LIPOCORTINS AND THE MECHANISM OF STEROID ACTION

The unexpected discovery in 1949 that cortisone dramatically reduced the symptoms of rheumatoid arthritis (Hench *et al.* 1949), heralded a new era in the treatment of inflammatory disease. Since that time steroids have become widely used in the therapy of many inflammatory conditions including MS, rheumatoid arthritis, asthma and psoriasis.

The anti-inflammatory steroids are all analogues of hydrocortisone, a naturally occurring steroid hormone released by the adrenal cortex and involved in glucose homeostasis. Extensive studies have shown that these compounds influence virtually every phase and component of the inflammatory and immune responses (see: Fauci 1979). One of the main actions of steroids is to prevent the accumulation of leukocytes at sites of inflammation and immune reactivity. Another important effect is their ability to inhibit the synthesis of, and cellular responsiveness to, many immune and inflammatory mediators such as IFNs, ILs, PGs, LTs, PAF, bradykinin, histamine and neutral proteases. In addition, steroids appear to influence many aspects of leukocyte function for example: cellular activation, proliferation, differentiation, phagocytosis and antigen processing, although many of these effects have been observed *in vitro* and it is not clear if they occur at physiologically/pharmacologically relevant concentrations *in vivo*.

As well as their ability to regulate immune and inflammatory responses, glucocorticoids also play an important physiological role in the homeostasis of

substrates for energy production. They have profound effects on carbohydrate, protein and fat metabolism which unfortunately are responsible for most of the side effects associated with steroid therapy. Acute dosage is generally well tolerated, but chronic treatment can result in the development of buffalo hump, moon face, a tendency to bruise easily, hypertension, osteoporosis, muscle wasting, skin thinning and glaucoma due to metabolic disturbances, and decreased resistance to infection caused by immunosuppression. Thus the use of these potent and very powerful drugs in chronic disease is often limited by the side effects which frequently arise from prolonged treatment (Flower & Dale 1989).

1.3.1 Early Studies on the Mechanism of Steroid Action (1971 - 1984)

Over the last two decades a great deal of work has been done to elucidate the mechanism by which steroids exert their anti-inflammatory actions. In 1971 it was discovered that the anti-inflammatory effects of aspirin and other NSAIDS were due to inhibition of cyclo-oxygenase, the enzyme responsible for synthesis of PGs from arachidonic acid (Vane 1971). Steroids were found to be inactive against cyclo-oxygenase (Flower *et al.* 1972), however several studies then demonstrated that glucocorticoids were able to block PG release (Herbaczynska-Cedro & Staszewska-Barczak 1974, Lewis & Piper 1975) but not the conversion of exogenous arachidonic acid (Gryglewski *et al.* 1975, Nijkamp *et al.* 1976). Subsequently, experiments utilising radio-labelled phospholipids determined that glucocorticoids were preventing eicosanoid production by inhibiting the release of arachidonic acid (Hong & Levine 1976, Blackwell *et al.* 1978). Since the rate limiting step in eicosanoid generation was thought to be the PLA_2 catalysed hydrolysis of arachidonic acid from membrane phospholipids, these studies suggested that steroids may be having a specific inhibitory effect on this enzyme.

At about this time it was realised that most, if not all, steroid actions are mediated indirectly via what is now known as the "classical pathway of steroid action" where binding of the steroid to a specific cytoplasmic receptor is followed by translocation of the activated steroid-receptor complex to the nucleus. Here it binds to specific sites on the DNA, initiating transcription of mRNA and the synthesis of new protein (Buller & O'Malley 1976, Baxter 1976, Munck & Leung 1977). These proteins are the effectors through which steroid actions are brought about, with different effects being mediated by different proteins. Discovery of the classical pathway prompted several workers to investigate whether glucocorticoids were inducing a protein inhibitor of PLA_2.

Subsequently it was demonstrated by a number of groups, using various *in vitro* systems, that inhibition of eicosanoid synthesis by steroids, correlated with and was dependent upon glucocorticoid receptor occupancy, and could be abolished by inhibitors of RNA and protein synthesis (Danon & Assouline 1978, Flower & Blackwell 1979, Di Rosa & Persico 1979, Russo-Marie *et al.* 1979, Hirata *et al.* 1980, Russo-Marie & Duval 1982). Each group then set about isolating and identifying the proteins that might be responsible for the PLA_2 inhibitory actions of the anti-inflammatory steroids.

Using the isolated guinea pig perfused lung, Flower, Blackwell and colleagues at the Wellcome Laboratories in Kent, demonstrated that steroids caused release of an anti-phospholipase factor which could be destroyed by boiling or proteolytic enzymes (Flower & Blackwell 1979). At the same time Di Rosa and co-workers at the University of Naples observed that glucocorticoid inhibition of PG production by rat peritoneal lavage cells was also mediated by release of a phospholipase inhibitory protein (Carnuccio *et al.* 1980). Collaborative studies by the Wellcome and Naples groups showed that the two factors were probably the same substance, a polypeptide with a molecular mass of about 15 kDa which was

named macrocortin (Blackwell *et al.* 1980). Partial purification of macrocortin from the peritoneal lavage fluid of steroid treated rats revealed several different peptides with anti-phospholipase activity, the most prominent species having molecular masses of 40 and 15 kDa (Blackwell *et al.* 1982). Further purification using a PLA_2 affinity column yielded a highly purified 41 kDa protein (Parente & Flower 1985a).

Another group working at the National Institute of Health in Bethesda, observed that prevention of neutrophil chemotaxis by steroids involved inhibition of PLA_2 and concluded that this was due to the induction of a protein inhibitor of the enzyme which appeared to have a molecular mass of approximately 40 kDa (Hirata *et al.* 1980) and was named lipomodulin (Hirata *et al.* 1981). Lipomodulin was purified to near homogeneity from conditioned media from primary cultures of rabbit neutrophils stimulated with glucocorticoids and the molecular mass of the predominant species was 40 kDa although others of 15 and 25-30 were also found (Hirata 1983).

Yet another group, headed by Russo-Marie at the Necker Hospital in Paris, were investigating the inhibitory effect of corticosteroids on PG release from cultured rat renomedullary cells. They found that abolition of eicosanoid production was due to inhibition of phospholipase and that this was caused by generation of a heat-labile, non-dialysable factor which they termed renocortin (Cloix *et al.* 1983). Partial purification of renocortin from the culture supernatants of dexamethasone treated cells revealed two species of 15 and 30 kDa and another of 45 kDa in the absence of serum (Rothhut *et al.* 1983).

Collaborative work by the three groups lead them to conclude that macrocortin, lipomodulin and renocortin were functionally identical and that the disparity in molecular weights was probably due to proteolysis. In 1984 the composite name lipocortin was agreed (Di Rosa *et al.* 1984).

1.3.2 The Lipocortins (1980 - 1987)

The lipocortins were defined by their discoverers as steroid-modulated anti-phospholipase proteins which might be responsible, in part, for the anti-inflammatory effects of the glucocorticoids (Di Rosa *et al.* 1984).

1.3.2.1 Inhibition of PLA_2

In most of the original experiments evidence for the inhibition of PLA_2 by lipocortins was only circumstantial. In 1981, Hirata was the first to demonstrate inhibition of the enzyme by purified lipocortin in a cell-free assay system. Using micellular phosphatidylcholine as a substrate, lipocortin was shown to inhibit porcine pancreatic, snake venom and bee venom PLA_2 enzymes in a concentration dependent manner (Hirata 1981). A further study (Hirata 1983) suggested that a stoichiometric complex was formed between enzyme and inhibitor, and that lipocortin altered the V_{max} of the reaction but not the K_m of the enzyme. Inhibition of the pancreatic enzyme by purified lipocortin was confirmed by other workers using pure phospholipid vesicles (Blackwell *et al.* 1982) or [^{3}H]oleate labelled *E. coli* (Rothhut *et al.* 1983).

The most likely target however, for down regulation of eicosanoid release by anti-phospholipase proteins is the calcium-dependent membrane-bound enzyme. Studies showing that lipocortins could prevent arachidonic acid release from rabbit neutrophil (Hirata *et al.* 1980) and rat renomedullary cell (Russo-Marie & Duval 1982) membranes, strongly suggested an inhibitory effect on endogenous PLA_2, and subsequently lipocortin inhibition of macrophage membrane PLA_2 was demonstrated directly (Blackwell 1983, Ghiara *et al.* 1984).

It was also observed that lipocortins could be phosphorylated by a cAMP-dependent protein kinase and that phosphorylation caused a decrease in anti-

phospholipase activity (Hirata 1981) which was reversible by treatment with alkaline phosphatase (Hirata *et al.* 1982). Phosphorylation/dephosphorylation of lipocortins was suggested to be a mechanism by which the anti-phospholipase activity of these proteins might be regulated *in vivo.*

1.3.2.2 Modulation by Steroids

Lipocortin-like activity was first detected *in vitro* in culture supernatants from steroid-treated neutrophils, peritoneal leukocytes and renomedullary cells and in the perfusion effluent of steroid-treated lungs (Hirata *et al.* 1980, Blackwell *et al.* 1980, Cloix *et al.* 1983). Detailed studies on the time course of steroid-induced release of anti-phospholipase proteins using rat peritoneal lavage cells, suggested a biphasic response to steroid treatment occurred in these cells, with an initial rapid release of existing pools after about 30 minutes, followed by a slower phase of resynthesis and further release after approximately 4-5 hours (Blackwell *et al.* 1980, Carnuccio *et al.* 1981). Furthermore, it was demonstrated that lipocortin secretion from peritoneal leukocytes was specific for glucocorticoids and did not occur in response to other steroids or non-steroid macrophage activation (Blackwell 1983). Human tissues were also found to synthesise and release lipocortins following *in vitro* treatment of embroyonic skin fibroblasts (Errasfa *et al.* 1985) and endometrium fragments (Gurpide *et al.* 1986) with dexamethasone.

In addition to these *in vitro* studies, steroid induction of anti-phospholipase proteins was also observed *in vivo.* Blackwell *et al.* (1982) showed that lipocortin-like activity in the peritoneal lavage fluid of rats was increased by injection of dexamethasone or hydrocortisone, but not if the animals were first treated with inhibitors of RNA/protein synthesis. Furthermore, the presence of much lower levels of anti-phospholipase proteins in the lavage fluid of Adx rats, coupled with the observation that treatment with ACTH induced release of the proteins in

normal but not Adx animals, suggested that lipocortin levels were normally regulated by endogenous glucocorticoids. In another study utilising immunoassay techniques, lipocortin-immunoreactivity was detected in all tissues of the rat and guinea pig and following treatment with dexamethasone, immunoreactivity was found to be increased in almost all organs except some gastrointestinal tissues (Flower 1984).

1.3.2.3 Anti-inflammatory Effects

Evidence that lipocortins were steroid modulated and possessed the ability to inhibit PLA_2 was followed by experiments showing that the purified proteins were able to mimic many of the anti-inflammatory effects of the glucocorticoids.

In vitro studies demonstrated that, like the glucocorticoids, lipocortins suppressed production of both cyclo-oxygenase and lipoxygenase derived mediators. In initial experiments crude extracts of anti-phospholipase proteins were found to inhibit thromboxane release from the guinea pig perfused lung (Blackwell *et al.* 1980), PGE_2 production by rat leukocytes (Di Rosa & Persico 1979, Blackwell *et al.* 1980, Carnuccio *et al.* 1980,1981) and renomedullary cells (Russo-Marie & Duval 1982), and the release of arachidonic acid from cultured fibroblasts (Hirata *et al.* 1981). Subsequently it was shown that partially purified lipocortin inhibited production of PGE_2 and LTB_4 by phagocytosing rat peritoneal leukocytes (Parente *et al.* 1984), and that the PLA_2-affinity purified protein blocked zymosan-induced release of lyso-PAF from these cells (Parente & Flower 1985a), effects which were reversed by a specific anti-lipocortin monoclonal, RM23. Moreover, studies demonstrating that the inhibitory effects of steroids on PG release from macrophages (Flower 1984) and human fibroblasts (Errasfa *et al.* 1985) could be prevented by lipocortin antibodies, suggested that lipocortin release occurred physiologically.

Consistent with their proposed function as second messengers of the anti-inflammatory effects of the glucocorticoids, lipocortins were demonstrated to be active *in vivo*, suppressing animal models of inflammation. Blackwell *et al.* (1982) observed that crude extracts of peritoneal lavage fluid from dexamethasone treated rats, inhibited fluid exudation and leukocyte accumulation associated with carrageenan-induced pleurisy, when injected into the pleural cavity. Partially purified lipocortins were shown to exhibit local anti-inflammatory properties in the rat carrageenan paw oedema model (Parente *et al.* 1984, Calignano *et al.* 1985).

Work by Hirata's group suggested that lipocortins might be implicated in some of the immunosuppressive actions of corticosteroids. Hirata and Iwata (1983) demonstrated that lipocortin was released by T lymphocytes and that the purified protein inhibited the proliferative response of thymocytes to mitogens such as phytohaemagglutinin and concanavalin A. Furthermore, they showed that whereas lipocortin antibody caused a selective loss of suppressor function, culture of thymocytes with lipocortin, inhibited proliferation of T helper cells and promoted the maturation of T suppressors suggesting that lipocortins had the ability to modulate T cell subset ratios. In other studies purified lipocortins were found to suppress the IgE response (Uede *et al.* 1983), inhibit natural killer activity and antibody-dependent cellular cytotoxicity (Hattori *et al.* 1983a), and block IL-2 production by T helper cells (Hirata 1984).

In addition, purified lipocortin was also shown to mimic other effects of steroids such as induction of differentiation (Hattori *et al.* 1983b), their protective action in reperfusion injury (Koltai *et al.* 1984) and teratogenic effects (Gupta *et al.* 1984).

1.3.2.4 Sequencing, Cloning and Relationships to Other Proteins

In 1986 workers at Biogen, Massachusetts, purified and partially sequenced a 37 kDa protein which was the major PLA_2 inhibitor present in rat peritoneal lavage fluid (Pepinsky *et al.* 1986). Using amino acid sequence information from the rat protein, the human gene was located in a U937 cDNA library, cloned and expressed in *E. coli*, resulting in the availability, for the first time, of large amounts of highly purified recombinant protein (Wallner *et al.* 1986). In a further study on anti-phospholipase proteins in human placental extracts, two different species of lipocortin were observed. One was identical to the recombinant protein and was named lipocortin I. The other which was of a similar molecular mass and shared approximately 50% sequence homology was labelled lipocortin II (Huang *et al.* 1986).

A suprise finding which emerged following the sequencing of lipocortins I and II (Pepinsky & Sinclair 1986, Huang *et al.* 1986) was that lipocortin I was identical with a protein termed p35, which was first isolated as an endogenous substrate for the epidermal growth factor receptor/kinase from A431 cells (Fava & Cohen 1984). Furthermore, lipocortin II was found to be the human analogue of p36 from bovine intestinal brush borders, a major substrate for the viral oncogene kinase $pp60^{v\text{-}src}$ (Gerke & Weber 1984). Moreover, it soon became apparent that calpactin II a protein with calcium, phospholipid and actin binding properties, thought to be involved in cytoskeletal biochemistry or vesicle secretion, was also identical to lipocortin I (Glenney *et al.* 1987).

Further studies by the Biogen group extended the known number of lipocortins to six (Pepinsky *et al.* 1988). Gradually it was realised that the lipocortins were structurally related to many other proteins which had been independently isolated from a variety of tissues and named according to their source and properties, such as the placental anti-coagulant proteins (Funakoshi *et*

al. 1987b), calelectrins (Geisow & Walker 1986), chromobindins (Creutz *et al.* 1987), endonexins (Geisow & Walker 1986), calcimedins (Smith & Dedman 1986) and synexin (Burns *et al.* 1989). The independent discovery, by several different groups, of proteins with apparently diverse biological functions, has resulted in a confused literature with many different names describing the same protein. Recently it has been proposed (Crumpton & Dedman 1990) that the name annexin (Geisow 1986) followed by the numbering system of the lipocortins (Pepinsky *et al.* 1988) be adopted.

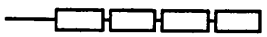
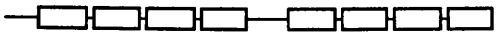
1.3.3 The Annexin Family of Proteins (1987 - To Date)

The annexins are an exceptionally homologous group of proteins, with different proteins sharing approximately 50% sequence identity, and interspecies identities for the same protein ranging from 85-98% (Pepinsky *et al.* 1988). To date twelve different members of the annexin family have been identified (Table II). The common structural feature present in all these proteins is a core region comprised of multiple copies of a 70 amino acid residue sequence. Within each of these repeats there is a very highly conserved 17 amino acid consensus sequence which was the first structural feature to be recognised as a common theme in these proteins (Kretsinger & Creutz 1986, Geisow *et al.* 1986). Most annexins have a molecular mass in the range 30-40 kDa and contain four copies of this motif (Saris *et al.* 1986, Pepinsky *et al.* 1988). Annexin VI which has a molecular mass of approximately 68 kDa has eight (Moss *et al.* 1988).

The core domain of each protein is preceded by an amino terminal sequence which although variable in length, is generally shorter and is unique to each protein. In many annexins the N-terminal region carries sites for phosphorylation by tyrosine (Glenney & Tack 1985, Pepinsky & Sinclair 1986) and serine-threonine kinases (Schlaepfer & Haigler 1988) and this region of annexins I and II has been

Table II

The annexin family of proteins

Annexin	M_r	Schematic Structure (N-terminal — Core — C-terminal)	Synonyms
I	38		Lipocortin 1, p35, Calpactin II, Chromobindin 9, GIF
II	38		Lipocortin II, p36, Calpactin I, Chromobindin 8, Protein I, PAP-IV
III	35		Lipocortin III, PAP III, 35-α Calcimedin
IV	35		Lipocortin IV, Endonexin I, Protein II, 32.5k Calelectrin, Chromobindin 4, PAP II, PP4-X, 35-β Calcimedin
V	35		Lipocortin V, IBC, PAP I, 35k Calelectrin, Endonexin II, PP4, VAC-α, 35-γ Calcimedin, Anchorin CII, Calphobindin I, Chromobindin 5
VI	68		Lipocortin VI, p68, p70, p73, 67k Calelectrin, Protein III, Chromobindin 20, 67k Calcimedin, Calphobindin II

VII	47		Synexin
VIII	37		VAC-β

p35, p36 - kinase substrates

p35 was originally isolated as the major substrate for the epidermal growth factor receptor/kinase from the A431 human carcinoma cell line (Fava & Cohen 1984). p36 was isolated from bovine intestinal brush borders as a substrate for the viral oncogene kinase $pp60^{v\text{-}src}$ (Gerke & Weber 1984). As kinase substrates these proteins are thought to be involved in the mitogenic signal transduction pathway and thus the regulation of cell growth and replication.

Calpactins

The calpactins were so named because of their calcium-dependent interaction with phospholipids and actin. Calpactin I is a heterotetramer composed of two 36 kDa heavy chains of annexin II and two 11 kDa light chains belonging to the S100 protein family (Glenney & Tack 1985). Calpactin I has been shown to promote fusion of adrenal chromaffin granules (Drust & Creutz 1988), to be of importance in exocytosis in permeabilised chromaffin cells (Ali *et al.* 1989) and to cause bundling of actin filaments (Ikebuchi & Waisman 1990). It has been suggested that these proteins play a role in membrane trafficking and may be involved in the control of secretion and cell motility.

Chromobindins

Chromobindins were identified in bovine adrenal medulla (Creutz *et al.* 1978) as a group of proteins associating with chromaffin granules in the presence of calcium and are thought to play a role in exocytosis (Creutz *et al.* 1987).

Calelectrins
The 35 kDa calelectrin was a calcium-binding protein originally purified from the electric organ of *Torpedo marmorata* (Walker 1982). Subsequently a family of Ca^{2+}-dependent membrane binding proteins was characterised by Sudhof *et al.* 1988.

Calcimedins
The calcimedins are five related Ca^{2+}-binding proteins which were isolated from smooth muscle homogenates by Moore and Dedman (1982). Recently the 67 kDa calcimedin has been shown to modify gating behaviour of the calcium release channel in skeletal muscle sarcoplasmic reticulum and thus has been proposed to be important in excitation-contraction coupling (Diaz-Munoz *et al.* 1990).

Proteins I, II and III
These proteins were originally identified in intestinal epithelium as constituents of the brush border cytoskeleton (Shadle *et al.* 1985).

Endonexins
Endonexins were first isolated from liver and adrenal medulla (Geisow *et al.* 1984). Endonexin II has been shown to have anticoagulant properties *in vitro* (Kaplan *et al.* 1988).

PAP
Placental anticoagulant proteins (PAPs) were isolated from human placenta and found to inhibit blood coagulation *in vitro* (Funakoshi *et al.* 1987a, Tait *et al.* 1988). They appear to do so by binding to anionic phospholipids thereby preventing binding of clotting factors important in blood coagulation. PAPs have been suggested to function as circulating components of the coagulation system.

IBC
Inhibitor of blood coagulation (IBC) has been shown to block blood coagulation *in vitro* and it has been proposed, may regulate the clotting cascade *in vivo* (Iwasaki *et al.* 1987).

VAC
Vascular anticoagulant (VAC) proteins isolated from human umbilical cord arteries (Maurer-Fogy *et al.* 1988).

PP4 and PP4-X
Placental proteins (PP) first isolated from human placenta (Bohn *et al.* 1985), also shown to have anticoagulant properties (Romisch *et al.* 1990).

Synexin
Synexin was originally isolated from bovine adrenal medulla as the result of a search for proteins regulating the fusion of granule membranes during exocytosis (Creutz *et al.* 1978). Recently it has been demonstrated that the protein can act as a voltage-sensitive calcium channel (Rojas & Pollard 1987, Pollard *et al.* 1990).

Anchorin CII
Isolated from chondrocyte membranes (Mollenhauer & von der Mark 1983), appears to mediate binding of collagen to the cell surface (Pfaffle *et al.* 1988).

GIF
Glycosylation inhibitory factor (GIF): a protein released from T cells thought to be involved in suppression of the IgE response (Uede *et al.* 1983).

p68
A Ca^{2+}-binding protein first identified as a component of lymphocyte plasma membranes (Davies *et al.* 1984, Crompton *et al.* 1988).

Annexin III
Annexin III has recently been found to be identical with inositol 1,2,-cyclic phosphate 2-phosphohydrolase, an enzyme of the phosphatidylinositol signalling pathway (Ross *et al.* 1990).

Annexins IX-XII
Annexins IX-XII are novel proteins which have recently been discovered in *Drosophila melanogaster* (XI and X; Johnston *et al.* 1990), bovine tissues (XI; Towle & Treadwell 1992) and *Hydra vulgaris* (XII; Schlaepfer *et al.* 1992).

shown to be particularly susceptible to proteolysis (Huang *et al.* 1987, Glenney & Tack 1985). In addition, loci for the dimerization of annexin I (Pepinsky *et al.* 1989) and the association of annexin II with the p11 protein to form calpactin I (Glenney 1986) are also located in this area.

A common property of all annexins is their ability to bind calcium and anionic phospholipids. They differ however from other calcium binding proteins such as the calmodulin family, in that they lack the classical "EF-hand" calcium binding motif (Kretsinger & Creutz 1986). Binding sites for calcium and phospholipids are located in the core region, but appear to be modulated by the N-terminal domain since both phosphorylation (Schlaepfer & Haigler 1987, Powell & Glenney 1987) and cleavage of the amino terminus (Ando *et al.* 1989) have been shown to influence binding affinities.

In addition to their proposed role as humoral mediators of the anti-inflammatory actions of the glucocorticoids, many other functions have been postulated for various members of the annexin family including regulation/mediation of: membrane trafficking, exocytosis and secretion; cell growth, replication and differentiation; the inositol phosphate signalling pathway; excitation-contraction coupling; cell surface collagen binding and phagocytosis. They have also been suggested to act as: structural proteins; circulating anticoagulants and voltage dependent calcium channels (for details and references see Table II). None of these proposed functions is universally accepted and few clues have arisen from studies on the distribution of these proteins. Annexins have been found in all eukaryotic organisms investigated so far including mammals, fruit flies (Johnston *et al.* 1990), sponges (Robitzki *et al.* 1990), hydra (Schlaepfer *et al.* 1992) and plants (Smallwood *et al.* 1990). However studies on their cellular localisation in vertebrates have shown that the various proteins are differentially distributed, with some, such as annexins II (Gould *et al.* 1984) and IV (Silva *et al.*

1986) confined to a few distinct cell populations, but others for example annexin VI (Silva *et al.* 1986) appearing to be ubiquitously distributed, indicating that the individual genes may be independently regulated (Pepinsky *et al.* 1988). It has been suggested that the core region of the annexins may carry a common function whereas the N-terminal might confer different biological properties to each protein. At present the precise function of any member of this protein family has yet to be established.

The recent availability of highly purified recombinant annexins and specific monoclonal and polyclonal antibodies to these proteins, has resulted in a great deal of new work on the potential role of annexins as second messengers of the anti-inflammatory actions of the glucocorticoids. The remainder of this chapter will review recent developments in this area. Since the name annexin was proposed after the initiation of this project and has not been unequivocally agreed upon, the term lipocortin will be retained throughout the rest of this thesis.

1.3.3.1 The Mechanism of PLA_2 Inhibition

Following the cloning of lipocortin I several studies demonstrated that the recombinant protein was able to inhibit PLA_2 *in vitro* (Wallner *et al.* 1986, Huang *et al.* 1986, Pepinsky *et al.* 1986, Peers *et al.* 1987) thus confirming the results of earlier work using partially purified preparations. In addition, Pepinsky *et al.* (1988) isolated and purified six different lipocortins (lipocortins I-VI) which were all shown to possess PLA_2 inhibitory activity. Since that time however the mechanism of PLA_2 inhibition by lipocortins has become highly controversial. In 1987, Davidson and colleagues investigating the inhibition of PLA_2 by calpactin I (lipocortin II) found that calpactin was unable to bind PLA_2 but did bind to the phospholipid substrate, and that whereas pre-incubation of calpactin with the enzyme had no effect, pre-incubation with excess substrate prevented inhibition.

Furthermore they demonstrated that enzyme inhibition was dependent upon the concentration of phospholipid and could be completely abolished by high substrate concentrations (Davidson *et al.* 1987). These workers concluded that inhibition of PLA_2 by calpactins/lipocortins was not due to a direct interaction of inhibitor and enzyme but was caused instead by the protein binding to phospholipids, thus depleting the substrate and blocking enzyme access. Two other groups working on the inhibition of PLA_2 by lipocortin I (Haigler *et al.* 1987) and a human monocyte lipocortin (Aarsman *et al.* 1987) reported similar findings and reached the same conclusion.

Although some workers claim to have observed inhibition of PLA_2 by lipocortins in the presence of excess substrate (Pepinsky *et al.* 1988) or using the phospholipid substrate phosphatidylcholine, for which the protein has a low affinity (Ahn *et al.* 1988, Moss *et al.* 1988), at present the balance of evidence suggests that in cell-free assay systems, lipocortins inhibit PLA_2 by binding to substrate phospholipids (Flower 1988, Davidson & Dennis 1989). Whether, and by what mechanism, lipocortins inhibit the phospholipase enzymes involved in regulation of eicosanoid release *in vivo* has not been established.

1.3.3.2 Steroid Induction and Secretion

Controversy has also recently arisen as to whether lipocortins are steroid modulated and whether they are secreted from cells - apparent pre-requisites for proteins proposed to be humoral mediators of glucocorticoid effects.

Most investigations on steroid inducibility have concentrated on lipocortin I. The promoter regions of the rat and human lipocortin I genes have been shown to contain a number of potential glucocorticoid recognition elements (Pepinsky *et al.* 1988, Kovacic *et al.* 1991) and a large number of studies have demonstrated increases in lipocortin I mRNA and/or protein following treatment of cells, tissues

or whole animals with glucocorticoids. Steroid induction of lipocortin I mRNA has been observed in several mouse cell lines (Wong *et al.* 1990), and induction of the protein in a rat epithelial cell line (Piltch *et al.* 1989) and differentiated U937 cells (Solito *et al.* 1991). Furthermore, increased levels of lipocortin I mRNA or protein have been detected in primary cultures of human peripheral blood monocytes (Wallner *et al.* 1986, Browning *et al.* 1990), human amnion cells (Mitchell *et al.* 1988), rat alveolar epithelial cells (Ambrose & Hunninghake 1990b), rat peritoneal macrophages (Browning *et al.* 1990) and cat trachea (Lundgren *et al.* 1988), in response to *in vitro* treatment with corticosteroids.

Induction of lipocortin I has also been observed *in vivo*. Several studies have shown that administration of glucocorticoids to rats induces lipocortin I in resident peritoneal leukocytes (Wallner *et al.* 1986, Smillie *et al.* 1989b, Solito *et al.* 1990, Peers *et al.* 1993). In humans, increased amounts of the protein have been found in peripheral blood mononuclear cells (Goulding *et al.* 1990) and bronchoalveolar lavage fluid (Smith *et al.* 1989a, Ambrose & Hunninghake 1990a) following anti-inflammatory steroid treatment. Moreover, studies on cells and tissues taken from Adx rats or animals treated with the glucocorticoid antagonist RU 38486 suggest that lipocortin I levels are normally modulated by endogenous corticosteroids (Vishwanath *et al.* 1992, Peers *et al.* 1993). There is also some evidence that lipocortins II and V may be steroid-regulated. Glucocorticoid induction of lipocortin II was detected *in vitro* in differentiated U937 cells (Solito *et al.* 1991) and *in vivo*, induction of both lipocortins II and V has been observed in rat peritoneal macrophages (Smillie *et al.* 1989b, Solito *et al.* 1990, Peers *et al.* 1993).

There have however been a substantial number of reports where the authors have found no change in lipocortin levels following steroid treatment despite, in some cases, observing steroid-mediated effects. Several studies have demonstrated

inhibition of eicosanoid release by glucocorticoids *in vitro,* independent of lipocortin induction (Hullin *et al.* 1989, Bienkowski *et al.* 1989, Piltch *et al.* 1989, Gebicke-Haerter *et al.* 1991). Furthermore, in a number of studies the authors were unable to detect steroid induction of lipocortin I protein or mRNA in various cell lines (Bienkowski *et al.* 1989, Isacke *et al.* 1989, Beyaert *et al.* 1990, Violette *et al.* 1990) and in primary cultures of fibroblasts, lymphocytes and macrophages (Bronnegard *et al.* 1988, Northup *et al.* 1988, Wong *et al.* 1991), endothelial cells (Hullin *et al.* 1989) and astrocytes (Gebicke-Haerter *et al.* 1991). In addition, no change was observed in lipocortin I levels in mouse peritoneal macrophages (Wong *et al.* 1991) and rat brain (Strijbos *et al.* 1991) following *in vivo* treatment with corticosteroids. Other studies have reported no induction by steroids of lipocortin II in cultured cell lines (Isacke *et al.* 1989, Beyaert *et al.* 1990) and lipocortin VI in endothelial cells (Hullin *et al.* 1989).

The reasons for these discrepancies are not clear. Recently it has been proposed that the presence of additional factors (Philipps *et al.* 1989, Browning *et al.* 1990) or a critical differentiation state (Browning *et al.* 1990, Solito *et al.* 1991) may be required for steroid induction. It has also been suggested that steroids may alter the subcellular localisation of lipocortins, causing translocation of protein from the cytoplasm to the cell surface (Smillie *et al.* 1989b, Browning *et al.* 1990, Goulding *et al.* 1990, Peers *et al.* 1993).

Debate over whether lipocortins are secreted has arisen partly due to early immunological studies which indicated that annexins were intracellular proteins (Geisow *et al.* 1984, Glenney *et al.* 1987, Zokas & Glenney 1987) and partly because all annexin genes sequenced so far appear to lack the hydrophobic signal sequence which is essential for entry into the classical secretory pathway. Some workers have been unable to detect secretion of lipocortins *in vitro* (Northup *et al.* 1988, Isacke *et al.* 1989, Frey *et al.* 1991). However other studies have

demonstrated release of lipocortins into the media of cultured cells both spontaneously (Pfaffle *et al.* 1988, Jacquot *et al.* 1990, Violette *et al.* 1990) and in response to steroids (Solito *et al.* 1991). Furthermore, there is evidence that lipocortins I and V are selectively secreted into human prostate fluid (Christmas *et al.* 1991). Recently it has been suggested that lipocortins may belong to a new class of proteins which exit the cell via a mechanism alternative to the classical pathway (Muesch *et al.* 1990).

1.3.3.3 Biological Activity

Early studies on partially purified lipocortins showed that they had anti-inflammatory actions consistent with their proposed role as second messengers of the glucocorticoids. Despite controversy over steroid-inducibility and the mechanism of PLA_2 inhibition, many recent studies have demonstrated that highly purified and recombinant lipocortins possess similar properties and are able to mimic many of the anti-inflammatory effects of both the early protein preparations and the steroids themselves.

Most work has been done on lipocortin I. Several studies have reported that recombinant human (rh) lipocortin I and other highly purified preparations have the ability to inhibit release of eicosanoid pro-inflammatory mediators *in vitro*. Prostacyclin production by human umbilical artery fragments (Cirino & Flower 1987a) and rat peritoneal leukocytes (Cirino & Flower 1987b), plus thromboxane generation by the guinea pig perfused lung (Cirino *et al.* 1987), were all found to be blocked by the recombinant protein, although Northup *et al.* (1988) found that lipocortin I purified from human placenta was unable to inhibit zymosan-induced arachidonic acid release from mouse peritoneal macrophages. As well as their effects on eicosanoid synthesis, naturally occurring and recombinant lipocortin I have been shown to suppress the generation of active oxygen metabolites by

human neutrophils (Stevens *et al.* 1988) and guinea pig alveolar macrophages (Maridonneau-Parini *et al.* 1989) and to mimic steroid-induced differentiation in human carcinoma cells (Violette *et al.* 1990).

Recombinant human lipocortin I has also been demonstrated to have anti-inflammatory activity *in vivo*. Several studies have shown that local administration of rh lipocortin I inhibits rat paw oedema induced by carrageenan (Miele *et al.* 1988, Cirino *et al.* 1989, Browning *et al.* 1990), or PLA_2 (Cirino *et al.* 1989), although other workers have observed no effect of placental lipocortin I on carrageenan paw oedema (Northup *et al.* 1988). In addition the recombinant protein has been demonstrated to block IL-1 elicited neutrophil accumulation in the mouse air pouch model (Perretti & Flower 1993) and to suppress migration of leukocytes into inflammatory lesions in the rat when given intravenously (Errasfa & Russo-Marie 1989). Interestingly, a nonapeptide fragment of lipocortin I corresponding to residues 246-254, termed antiflammin, has also been shown to exhibit PLA_2 inhibitory activity and *in vivo* anti-inflammatory actions (Miele *et al.* 1988, Ialenti *et al.* 1990, Perretti *et al.* 1991) but conflicting reports do exist (van Binsbergen *et al.* 1989, Marki *et al.* 1991).

Fewer recent studies have been performed on the immunological properties of lipocortin I, although Goulding and Guyre (1988) have shown that rh lipocortin I impairs erythrocyte-antibody rosette formation by human leukocytes and recombinant mouse lipocortin I has been found to suppress the mitogenic response of murine splenic mononuclear cells (Sakata *et al.* 1990).

Little information is available on the biological activity of other members of the lipocortin family, however human lipocortin II has recently been cloned and has been demonstrated in one study to have similar inhibitory effects to lipocortin I on PGE_2 release by macrophages and on carrageenan paw oedema (Parente *et al.* 1990). In addition, Russo-Marie's group have purified, but not fully characterised,

several lipocortins possessing anti-inflammatory properties both *in vitro* (Fradin *et al.* 1988, Errasfa *et al.* 1988, Errasfa & Russo-Marie 1989, Comera *et al.* 1990) and *in vivo* (Errasfa & Russo-Marie 1989), the most extensively studied of which is a 32 kDa protein that appears to be lipocortin V (Browning *et al.* 1990).

Further evidence for the role of lipocortins in corticosteroid action has come from studies demonstrating that neutralising antibodies to lipocortin I are able to block glucocorticoid effects in several systems. *In vitro*, specific monoclonal antibodies raised against lipocortin proteins have been shown to prevent glucocorticoid inhibition of eicosanoid release from neutrophils (Fradin *et al.* 1988), respiratory glycoconjugate secretion by cat trachea (Lundgren *et al.* 1988) and PLA_2 activation by IL-1 in human fibroblasts (Solito & Parente 1989). *In vivo* administration of anti-lipocortin I antibodies reverses dexamethasone inhibition of IL-1-elicited neutrophil accumulation in the mouse air pouch (Perretti *et al.* 1992) and the anti-inflammatory effect of dexamethasone in the rat carrageenan paw oedema model (Duncan *et al.* 1993), findings which suggest that lipocortins may play a physiological role in mediating the anti-inflammatory actions of steroids.

The use of steroids for the treatment of chronic inflammatory diseases is frequently limited by their side effects. As potential mediators of the anti-inflammatory actions of corticosteroids, lipocortins have been the subject of intense interest, as they represent a possible mechanism through which the beneficial effects of steroids might be selectively brought about and thus may open up opportunities for the development of new therapies free of the major side effects associated with steroid treatment. At present however, it is not clear if any, some or all members of the annexin family of proteins are the steroid-regulated, phospholipase-inhibitory, second messengers of anti-inflammatory steroid action,

that the lipocortins were initially proposed to be.

1.4 AIMS

Corticosteroids are frequently used in the treatment of MS and are also effective in suppressing the animal counterpart, EAE. It is conceivable that the beneficial effects of steroids in these diseases may be mediated, in part, via the induction of lipocortins. Thus the aims of this project were to:

1) Determine whether the potential exists for lipocortins to be involved in these diseases by investigating the occurrence of lipocortins in the CNS of MS patients and EAE-diseased rats.
2) Shed light on the function of lipocortins in the CNS by studying their cellular distribution in MS and EAE.
3) Investigate the involvement of lipocortins in the mechanism of steroid action by studying the influence of exogenous and endogenous steroids on lipocortin levels in the CNS during EAE.

It is hoped that such a study will provide insight into the role of lipocortins in the physiological and pharmacological regulation of inflammatory CNS disease.

MATERIALS AND METHODS

2.1 HUMAN CNS SAMPLES

2.1.1 CSF

CSF samples from patients with a variety of diseases were obtained from Mr D. Marshall, Pathology Laboratory, Royal United Hospital, Bath. Samples had been collected within the previous week and were stored at 4°C. CSF protein concentrations plus erythrocyte and leukocyte counts were also supplied and for most patients a final diagnosis was provided from hospital records by Dr J. Lever.

2.1.2 CSF Supernatants

Cell-free CSF supernatants from patients with MS and other neurological diseases were supplied by Professor A.N. Davison, Institute of Neurology, London and were stored at -20°C.

2.1.3 CNS Tissue

Samples of post-mortem human brain and spinal cord were provided by Dr J. Newcombe, The Multiple Sclerosis Society Tissue Bank, Institute of Neurology, London. Tissues were supplied in coded form, from six control cases without neurological disease and seven patients diagnosed clinically as having MS. The clinical diagnosis of MS was subsequently confirmed by routine histological staining of CNS tissue with haematoxylin & eosin (H & E) and Oil-Red-O, which revealed the presence of inflammatory infiltrates and demyelinated plaques with axon sparing which are characteristic of the disease. The average age of the subjects, duration of clinical disease and time elapsed between death and freezing of the CNS tissue are shown in Table III. Medical records indicated that none of the patients with MS had received steroid therapy within two years of death. No information was available for control cases.

Table III

Age, disease duration and death to freezing interval for MS and control cases from which post-mortem CNS tissue was obtained.

	MS	Control
Number of cases	7	6
Age (years)	50 (33-74)	38 (20-50)
Duration of clinical disease (years)	18 (0.75-49)	N\A
Death to freezing interval (hours)*	41 (27-82)	20 (9-33)

Figures for age, duration of disease and death to freezing interval are means, range is given in parenthesis. * Time elapsed between death of subjects and freezing of CNS tissue sample.

Small areas of CNS tissue were dissected, prepared and classified by Dr Newcombe as described below. MS cases were dissected to provide samples of plaque tissue, macroscopically normal appearing white matter adjacent to a plaque, apparently uninvolved white matter remote from plaque areas and grey matter. Following histological staining, four of the plaques exhibited perivenular inflammation, hypercellularity and macrophages containing Oil-Red-O-positive degenerating myelin and these were thus considered to be actively demyelinating lesions. Two hypocellular Oil-Red-O-negative demyelinated plaques were classified as chronic plaques. No positive Oil-Red-O staining or myelin loss was detected microscopically in MS white matter samples. Control samples of white and grey matter were obtained by dissection from corresponding areas of the brain and spinal cord of normal controls.

Each tissue block was cut into two pieces of approximately 1 cm^3, one piece was placed in OCT compound (Gurr, BDH) on a cork disc then snap-frozen in isopentane cooled on liquid nitrogen for immunohistochemistry, the other was frozen at -70°C prior to Western blotting.

2.2 RAT CNS SAMPLES

2.2.1 Animals

Inbred male Lewis rats were used in all experiments and either obtained from the University Animal House breeding colony or purchased from Bantin and Kingman (Hull). The rats were housed 5-6 per cage, in temperature controlled rooms (22 ± 2°C) on a 12 hour light /dark cycle (light period: 6am-6pm) with free access to Labsure CRM rat diet and tap water. Cages containing severely paralysed animals had food pellets soaked in water placed inside the cage to

prevent death due to starvation and dehydration. Both in-house and externally purchased animals were randomly grouped and placed in the experimental room at least one week prior to the beginning of an experiment.

2.2.2 EAE

2.2.2.1 Induction of EAE

EAE was induced using an inoculum comprising guinea pig spinal cord, Freund's incomplete adjuvant (Difco) and sterile 0.01 M phosphate buffered saline (PBS) pH 7.2, in the ratio 1:1:1 plus 10 mg/ml killed *Mycobacterium tuberculosis* H37 RA (Difco). For preparation of the inoculum PBS was added to the guinea pig spinal cord which was then chopped with scissors and briefly homogenised by repeated passage through a 1 ml syringe. Following the addition of *M. tuberculosis* and Freund's incomplete adjuvant, the mixture was shaken vigorously and then emulsified by repeated passage between two 20 ml syringes connected by a short length of tubing. This was continued until the inoculum reached a thicker consistency and did not disperse when a drop was floated in a beaker of water. For the induction of EAE, rats weighing 200-250 g received 0.1 ml of this inoculum in each hind foot pad.

In some experiments control animals were injected with an inoculum in which the guinea pig spinal cord was substituted with sterile saline. Thus the inoculum consisted of an emulsion of Freund's incomplete adjuvant and sterile saline 1:2 plus 10 mg/ml *M. tuberculosis.* These were termed CFA controls.

2.2.2.2 Assessment of EAE

Following inoculation animals were weighed and examined daily for signs of EAE. The severity of disease was assessed and graded by assigning a numerical

score from 0-4 using the following system. Rats with no signs of disease were scored zero. If from day 7 post-inoculation (PI) onward but before the appearance of physical symptoms, the rats showed a small weight loss (1-2 g) for two consecutive days or a larger weight loss (>2 g) for one day they were classified as exhibiting disease-related weight loss. These were also scored zero. Animals were considered to have a flaccid tail and assigned a score of 1 if the tail was immobile and drooping when they were raised briefly by the base of the tail. Hind limb weakness was indicated by an ataxic gait and scored 2. Rats with only one leg completely paralysed, or both legs moving but neither functional were categorised as having partial hind limb paralysis and scored 3. A score of 4 was assigned to cases of complete hind limb paralysis.

Mean numerical scores were used to assess disease severity before and after experimental treatments. The significance of differences between treatment and control groups was assessed using the Mann-Whitney U test, with the Bonferroni Correction for multiple comparisons.

2.2.3 Administration of Drugs and Surgery

All surgery and administration of drugs were performed by the University Animal House staff.

2.2.3.1 Corticosterone

Corticosterone (Sigma) was suspended in approximately 0.2 ml arachis oil and administered subcutaneously (sub. cut.) to rats inoculated for EAE at a dose of 10 or 50 mg/kg body weight. EAE-inoculated vehicle controls received arachis oil only. Dosing was commenced either on the first day of total paralysis or on the first day of complete recovery from symptoms. In some experiments the rats were dosed twice (morning and afternoon) and killed the following morning, in other

experiments they were dosed 3 times (morning, afternoon and the following morning) and CNS and blood samples collected two hours after the last dose.

2.2.3.2 Dexamethasone

EAE-inoculated animals were dosed sub. cut. with 0.5 or 1 mg/kg dexamethasone sodium phosphate (Oradexon-Organon) diluted in approximately 0.2 ml sterile PBS. EAE-inoculated controls received vehicle only. The rats were dosed three times, twice on the first day of paralysis (morning and afternoon) and again the following morning. CNS and blood samples were collected two hours later.

2.2.3.3 RU 38486

The glucocorticoid antagonist RU 38486 (mifepristone; supplied by Dr R. Deraedt, Roussel UCLAF, France) was suspended in an aqueous solution containing 1% ($^{W}/_{V}$) carboxymethyl cellulose, sodium salt, (Sigma) and 0.05% ($^{V}/_{V}$) Tween 80 (Sigma). EAE-inoculated rats were dosed orally twice daily on days 10-14 PI inclusive with 20 mg/kg RU 38486 in approximately 0.2 ml vehicle. Controls which had also been inoculated for EAE received vehicle only. In addition, normal animals were dosed with either RU 38486 or vehicle for five days according to the same schedule. CNS tissue and blood samples were collected the day after the last dose, day 15 PI for EAE-inoculated rats.

2.2.3.4 Adrenalectomy

Rats weighing 180-200 g were anaesthetised by intraperitoneal injection with 3.3 ml/kg of an aqueous solution containing 1.25 mg/ml midazolam (Roche) plus 0.79 mg/ml fentanyl citrate and 2.5 mg/ml fluanisone (Hypnorm, Janssen) (Flecknell 1987). Under aseptic conditions a small midline incision was made in

the dorsum, through which both adrenal glands were removed. The incision was then sealed with Michel clips. Sham-operated controls were subjected to the same procedure except that the adrenals were not removed. Adx rats were maintained on 0.9% ($^{w}/_{v}$) saline. Both sham-operated and Adx animals were allowed 8-12 days to recover before inoculation for EAE.

2.2.4 Sera

Anaesthesia and the collection of blood samples were carried out by the University Animal House technicians. To limit fluctuations in corticosterone due to circadian variation, samples were always collected in the morning when plasma corticosterone levels are at their lowest (D'Agostino *et al.* 1982). If the experiment was to be continued following blood sampling, the animals were anaesthetised with halothane, 1 ml of blood was collected by cardiac puncture and the rats were then allowed to recover. If CNS samples were also to be taken the animals were rendered unconscious by carbon dioxide (CO_2) inhalation, 2 ml of blood was collected by cardiac puncture and the animals were then asphyxiated.

Blood samples were allowed to clot at 4°C and then centrifuged at 370 g for 10 min at 4°C and the serum collected. The sera were then clarified by a further centrifugation step and the supernatants stored at -20°C.

2.2.5 CSF

Rats were asphyxiated with CO_2, exsanguinated and an incision made at the base of the skull to expose the meninges covering the cisterna magna. A CSF needle (Figure 2) attached to a length of translucent vinyl tubing was then inserted through the membrane into the cavity. Spinal fluid was drawn off by gentle suction on the tubing which was then clamped off and the CSF transferred to chilled vials.

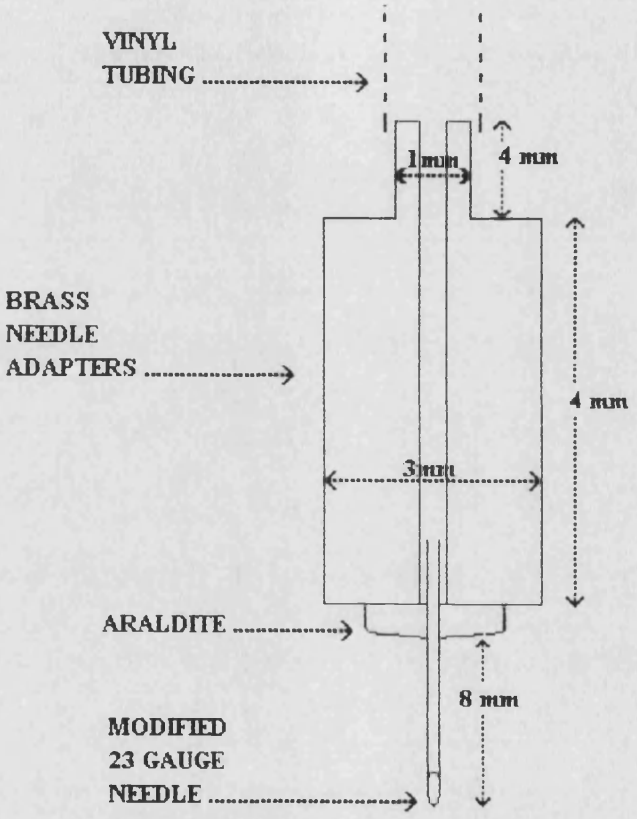


Figure 2. CSF needle
Brass needle adapters were made by Mr G. Venn, Science Schools Workshop. At one end of the adapter a 1 cm length of a 23 gauge needle (diameter 0.6 mm, Sherwood) with the bevel filed to approximately half length was fixed with Araldite adhesive. Translucent vinyl tubing (internal diameter 1.0 mm, external diameter 2.0 mm, Portex) was attached to the other end.

2.2.5.1 Enumeration of cells in CSF

CSF leukocyte and erythrocyte numbers were determined using a Fuchs-Rosenthal chamber with a ruled area consisting of 25 one millimetre squares with a depth of 0.2 ml. The number of cells in 25 squares was counted and the cell count per ml of CSF calculated using the formula:

$$\text{Number of cells per ml} = \text{Number of cells in 25 squares} \times 200$$

In initial experiments cell counts were performed on unstained CSF and red and white cells were differentiated on the basis of morphology and size. In later experiments a total cell count was performed on undiluted CSF and a leukocyte count by diluting 1:1 with white cell counting fluid. The number of erythrocytes was then calculated by subtracting the white cell count from the total. Samples containing $>1 \times 10^6$ erythrocytes per ml were discarded.

2.2.6 CNS Tissue

Rats were asphyxiated with CO_2 and exsanguinated. For removal of the cervical spinal cord a midline incision was made from the head down the back, the skin eased apart and some of the muscle tissue cut away. After piercing the membrane covering the cisterna magna the dorsal cervical vertebrae were removed using bone cutters and the first 3 cm of spinal cord (which in a 200-250 g rat corresponds approximately to the cervical section) were dissected out, washed in PBS and blotted dry. In most experiments the cord was then bisected down the midline, one half was immediately frozen at -20°C prior to Western blotting, the other was placed cut side down on histology card and transferred to fixative

solution. In one set of experiments the cerebellum was also removed and prepared as described above.

2.3 SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND IMMUNOBLOTTING

The techniques of SDS-PAGE and immunoblotting were performed using a method already established in our laboratory.

2.3.1 Preparation of Samples and Standards

To ensure solubility of lipocortins, ethylene diamine tetraacetic acid, disodium salt, dihydrate (EDTA, Sigma) was added to all samples. EDTA chelates calcium ions and has been shown to prevent calcium-dependent binding of lipocortins to phospholipids thus releasing the protein from cell membranes (Cirino & Flower 1987b, Klee 1988).

2.3.1.1 Human and Rat CSF

To each 60 µl aliquot of CSF, 6 µl of 100 mM EDTA was added to give a final concentration of 11 mM EDTA. Samples were then frozen and thawed five times to lyse cells, centrifuged at 11,000 g for 5 min to remove cell debris and the supernatants collected.

2.3.1.2 Human CSF Supernatants

Human CSF supernatants which had been stored at -20°C were thawed and 6 µl of 100 mM EDTA was added to each 60 µl sample to give a final concentration of 11 mM EDTA.

2.3.1.3 Rat CSF Supernatants

Freshly collected rat CSF samples were spun at 11,000 g for 5 mins to pellet the cells and the supernatants were collected.

2.3.1.4 Human and Rat CNS Tissue

CNS tissue samples which had previously been stored at -20°C were thawed and weighed. Chilled homogenisation medium comprising 10 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulphonic acid, sodium salt (HEPES, Sigma), 5 mM EDTA, 100 mM sodium chloride (Sigma) and 200 µM phenylmethylsulphonyl fluoride (PMSF, Sigma) pH 7.6 (Rothhut *et al.* 1987), was added to each sample at a rate of 0.5 ml per 100 mg of tissue. The samples were then chopped using scissors and homogenised on ice by repeated passage firstly through a syringe alone and then through 19 and 21 gauge needles. The homogenates were frozen and thawed twice, centrifuged at 19,000 g for 30 mins at 4°C and the supernatants collected.

Supernatants were assayed for total protein using the method of Bradford (1976). Sample aliquots of 10 or 20 µl were diluted to 100 µl with distilled water. Protein standards were prepared by dissolving bovine serum albumin (Sigma) at 1 mg/ml in distilled water and then diluting to provide 100 µl of standards ranging from 0.2-1.0 mg/ml. Protein reagent was prepared in advance by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Sigma) in 50 ml of ethanol (Fisons), 100 ml of 85% orthophosphoric acid (Fisons) was then added and the solution diluted to one litre with distilled water and shaken thoroughly. Five millilitres of protein reagent were added to 100 µl of duplicate samples and standards, and mixed thoroughly by vortexing. After two min the absorbence at 595 nm was measured in a Pye Unicam PU8610 spectrophotometer against a zero protein blank prepared from 100 µl distilled water and 5 ml of protein reagent. The protein concentration

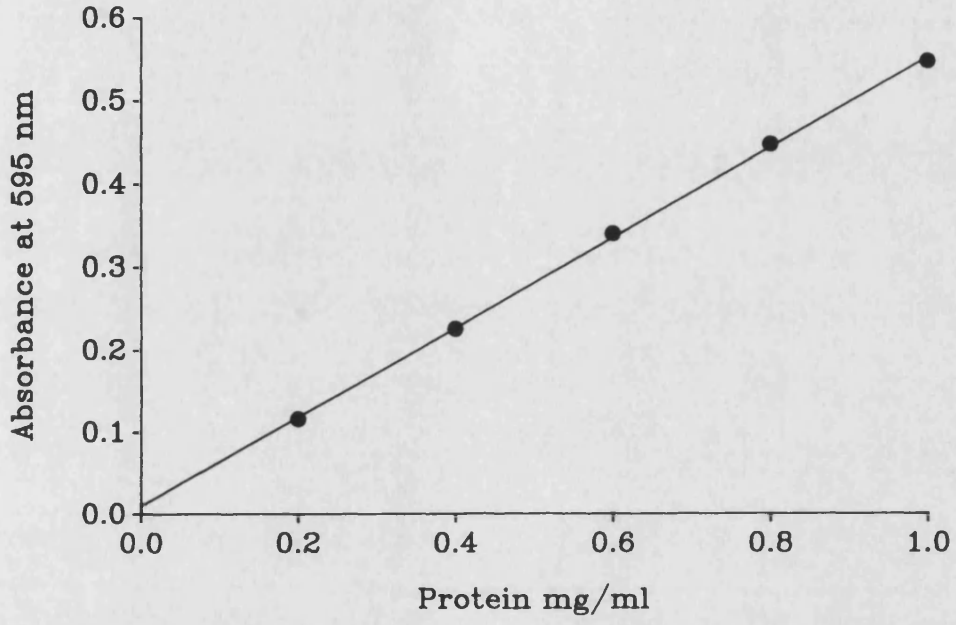


Figure 3. A typical standard curve obtained for the Coomassie G-250 protein assay

of CNS supernatants was calculated from a standard curve obtained by plotting protein concentration of standards against absorbence. A typical standard curve is shown in Figure 3. Each supernatant was then diluted to 2 mg/ml with homogenisation medium.

2.3.1.5 Lipocortin I Standard

Recombinant human lipocortin I (Wallner *et al.* 1986) was supplied by Dr J. Browning, Biogen Research Corporation, USA. For use as a standard in SDS-PAGE, rh lipocortin I was diluted to an appropriate concentration in homogenisation medium. Unfortunately standards for lipocortins II, IV and V were not available.

In each case following the treatment described above the samples or standards were then mixed in the ratio 1:1 with a sample buffer comprising 0.1 M Tris(hydroxymethyl)aminomethane (TRIS, Sigma), 0.1 M N,N-bis(2-hydroxyethyl)glycine (BICINE, Sigma), 2% ($^{W}/_{V}$) sodium dodecyl sulphate (SDS, BDH), 5% ($^{V}/_{V}$) 2-mercaptoethanol (Fisons), 10% ($^{W}/_{V}$) sucrose (BDH) and 0.05% ($^{W}/_{V}$) bromophenol blue (BDH), heated in a boiling water bath for 5 min and stored at -20°C prior to SDS-PAGE.

2.3.1.6 Molecular Mass Markers

Pre-stained high molecular mass markers for immunoblotting were obtained from Sigma (code: SDS-7B, range: 180,000-26,600 kDa). Following the addition of 0.8 ml of sample buffer (without bromophenol blue) per vial, the markers were boiled for 5 min and then stored in aliquots at -20°C. Unstained low molecular mass markers for use on protein stained gels were prepared in the same way (code: SDS-7, range: 66,000-14,200 kDa, Sigma).

2.3.2 SDS-PAGE

SDS-PAGE was performed essentially as described by Laemmli (1970) using a "Mighty Small II" vertical slab gel system (Hoefer Scientific Instruments) according to the manufacturers instructions.

Ten percent acrylamide resolving gels (82 x 60 x 1.5 mm) were prepared from stock solutions of 30% ($^{W}/_{V}$) acrylamide/0.8% ($^{W}/_{V}$) NN'-methylenebisacrylamide (electrophoresis grade, BDH) and 1 M TRIS/BICINE and polymerised chemically by the addition of NNN'N'- tetramethylethylenediamine (TEMED, Sigma) and freshly prepared 10% ($^{W}/_{V}$) ammonium persulphate (BDH) solution. Final concentrations in the gel were: 10% ($^{W}/_{V}$) acrylamide, 0.27% ($^{W}/_{V}$) NN'-methylenebisacrylamide, 0.1 M TRIS, 0.1 M BICINE, 0.1% ($^{W}/_{V}$) SDS, 0.09% ($^{W}/_{V}$) ammonium persulphate and 0.1% ($^{V}/_{V}$) TEMED. Immediately after pouring, 0.3 ml of 50% ($^{V}/_{V}$) ethanol was gently layered onto the surface of each gel. When set, the gels were rinsed with distilled water, wrapped in polythene and stored at 4°C for up to one week.

The electrode buffer was also made from stock solutions and comprised 0.1 M TRIS/BICINE and 0.1% ($^{W}/_{V}$) SDS. Stacking gels containing 7.5% acrylamide were prepared immediately before use by mixing acrylamide/NN'-methylenebisacrylamide stock solution, distilled water and electrode buffer in the ratio 1:1:2 and were also polymerised with TEMED and ammonium persulphate. The final concentrations in the stacking gel were: 7.5% ($^{W}/_{V}$) acrylamide, 0.2% ($^{W}/_{V}$) NN'-methylenebisacrylamide, 0.05 M TRIS, 0.05 M BICINE, 0.05% ($^{W}/_{V}$) SDS, 0.07% ($^{W}/_{V}$) ammonium persulphate and 0.7% ($^{V}/_{V}$) TEMED. Sample wells were formed in the stacking gel by means of a comb device suspended above the resolving gel. Stacking gel was poured beneath the comb and when set the comb was removed and the chamber filled with electrophoresis buffer. Wells were loaded with 5-20 µl of prepared samples or standards using a Hamilton syringe.

The gels were run at a constant current of 15 mA/gel until the samples had passed through the stacking gel (approximately 7 min) and then at 35 mA/gel until the bromophenol blue dye front reached the bottom of the gel (approximately 1 hr).

2.3.3 Staining Gels for Protein

Gels were stained for total protein using PAGE Blue 83. Following overnight incubation with an aqueous solution containing 0.2% ($^{W}/_{V}$) PAGE Blue 83 (BDH), 50% ($^{V}/_{V}$) methanol (Fisons) and 10% ($^{V}/_{V}$) acetic acid (Fisons), the proteins were visualised by destaining the background with several changes of a solution comprising 20% ($^{V}/_{V}$) methanol and 10% ($^{V}/_{V}$) acetic acid in distilled water.

2.3.4 Immunoblotting

Electrophoretic transfer of proteins from unstained gels to nitrocellulose membranes was performed essentially by the method of Towbin *et al.* (1979), using a Transphor electrophoresis tank (Hoefer Scientific Instruments) according to the manufacturers instructions. The electrode buffer was prepared from a stock solution of 0.25 M TRIS and 1.92 M glycine (Sigma) and the final solution comprised 25 mM TRIS, 192 mM glycine and 20% ($^{V}/_{V}$) methanol. Proteins were transferred to 0.45 μm nitrocellulose membranes (Schleicher & Schuell) at a constant voltage of 80 volts for 1 hr. Blots were often stored frozen at this point.

For the detection of lipocortins after protein transfer, membranes were subjected to the following procedure:

1) Incubated with 3% ($^{W}/_{V}$) milk powder for 1 hr to block remaining protein binding sites
2) Probed overnight with primary anti-lipocortin antibody. See Table IV

Table IV

Primary antibodies used for immunoblotting

Code	Type	Raised against	Species specificity	Known cross reactivities within the lipocortin family	Dilution
842	Rabbit polyclonal	Recombinant human lipocortin I	Human and rat	Lipocortin III	1:5000
774	Rabbit polyclonal	Native human lipocortin II	Human and rat	Lipocortin I	1:5000
179	Rabbit polyclonal	Native bovine lipocortin IV	Human but not rat	-	1:5000
890	Rabbit polyclonal	Native rat lipocortin V	Human and rat	-	1:5000
mAb 1B	Mouse monoclonal	Recombinant human lipocortin I	Human	-	1:500
Ab-1	Rabbit polyclonal	Recombinant human lipocortin II	Human	-	1:1000

All antibodies except Ab-1 were generously supplied by Dr J. L. Browning, Biogen Research Corporation, USA. Crossreactivity data for these antibodies have been reported by Pepinsky *et al* (1988). Ab-1 was kindly supplied by Dr L. Parente, Sclavo Research Centre, Siena, Italy.

3) Washed 2 x 15 min
4) Incubated for 1 hr with a 1:1000 dilution of goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) if the primary antibody was a rabbit polyclonal, or sheep anti-mouse IgG-horseradish peroxidase conjugate if the primary antibody was a mouse monoclonal
5) Washed 1 x 15 min
6) Washed 1 x 15 min in PBS
7) Incubated with 0.05% ($^{w}/_{v}$) filtered 3,3'-diaminobenzidine tetrahydrochloride (DAB, Aldrich) plus 0.02% ($^{w}/_{v}$) hydrogen peroxide (H_2O_2, BDH) in PBS for approximately 10 min until the colour was fully developed
8) Washed 2 x 5 min in distilled water

All incubations were carried out at room temperature in PBS containing 0.1% ($^{v}/_{v}$) Tween 20 (Sigma) (PBS-Tween) unless otherwise stated. The incubation volume was always 50 ml except for steps using antibodies where it was reduced to 25 ml to conserve reagents.

2.3.5 Comparison of Immunoblotting Detection Methods

Due to the very low levels of lipocortin detected in initial studies, an experiment was performed to compare the sensitivity of various putative methods of signal enhancement (Stott, 1989). Several gels loaded with a range of concentrations of rh lipocortin I standard (0.05-100 ng/lane) were prepared under identical conditions. Immunoblotting was initially performed as described above but following incubation with lipocortin I antisera, the primary antibody was detected using one or a combination of the following methods: peroxidase

conjugate, streptavidin-biotin complex, peroxidase-anti-peroxidase, and nickel and cobalt enhancement.

Antibody reagents for each technique were used at the manufacturers recommended working dilution and incubations were always performed at room temperature in PBS-Tween unless otherwise stated. All blots were blocked, probed with lipocortin antisera (842) and washed twice for 15 min in PBS-Tween as described previously. Detection was then completed using one of the protocols listed below.

2.3.5.1 Streptavidin-biotin Complex (Coggi *et al.* 1986).

1) Incubated 1 hr with biotinylated donkey anti-rabbit IgG (Amersham) diluted 1:400
2) Washed 2 x 15 min
3) Incubated 30 min with streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:3000
4) Washed 1 x 15 min
5) Washed 1 x 15 min in PBS
6) Incubated with 0.05% ($^{w}/_{v}$) filtered DAB plus 0.02% ($^{w}/_{v}$) H_2O_2 in PBS until colour developed
7) Washed 2 x 5 min in distilled water

2.3.5.2 Peroxidase-anti-peroxidase Complex (Frazer and Wisdom 1985).

1) Incubated 1 hr with goat anti-rabbit IgG (Sigma) diluted 1:200
2) Washed 2 x 15 min
3) Incubated 1 hr with rabbit peroxidase-anti-peroxidase complex (PAP, Sigma) diluted 1:200
4) Washed 1 x 15 min

5) Washed 1 x 15 min in PBS
6) Incubated with 0.05% ($^{w}/_{v}$) filtered DAB plus 0.02% ($^{w}/_{v}$) H_2O_2 in PBS until colour developed
7) Rinsed 2 x 5 min in distilled water

2.3.5.3 Nickel and Cobalt Enhancement (De Blas and Cherwinski 1983)

Blots were probed using one of the techniques described above but after incubation with the final peroxidase conjugated reagent and rinsing in PBS-Tween and PBS, immunoreactive bands were visualised by the following method:

1) A 0.05% ($^{w}/_{v}$) solution of DAB in PBS was prepared and filtered
2) An aqueous solution of 1% ($^{w}/_{v}$) cobalt chloride ($CoCl_2.6H_2O$, Sigma) and 1% ($^{w}/_{v}$) ammonium nickel sulphate ($[NH_4]_2SO_4.NiSO_4.6H_2O$, BDH) was prepared (Ni/Co)
3) Ni/Co solution was added dropwise to stirring DAB solution, 3 ml per 100 ml DAB
4) Membranes incubated in 50 ml Ni/Co/DAB solution for 10 min
5) Blots then transferred to an identical solution containing 0.02% ($^{w}/_{v}$) H_2O_2 for approximately 2 min until colour fully developed
6) Washed 2 x 5 min distilled water

The combination of PAP complex with Ni/Co enhancement was found to give maximum sensitivity (approximately 0.5-1 ng lipocortin/lane) with acceptable background staining and was approximately 10 times more sensitive than the original peroxidase conjugate technique (Figure 4). For titration of reagents used in the PAP method, two blots containing 50 ng of rh lipocortin I in each lane were prepared. After probing with the primary antibody one blot was divided into strips which were incubated with a range of concentrations of the secondary reagent

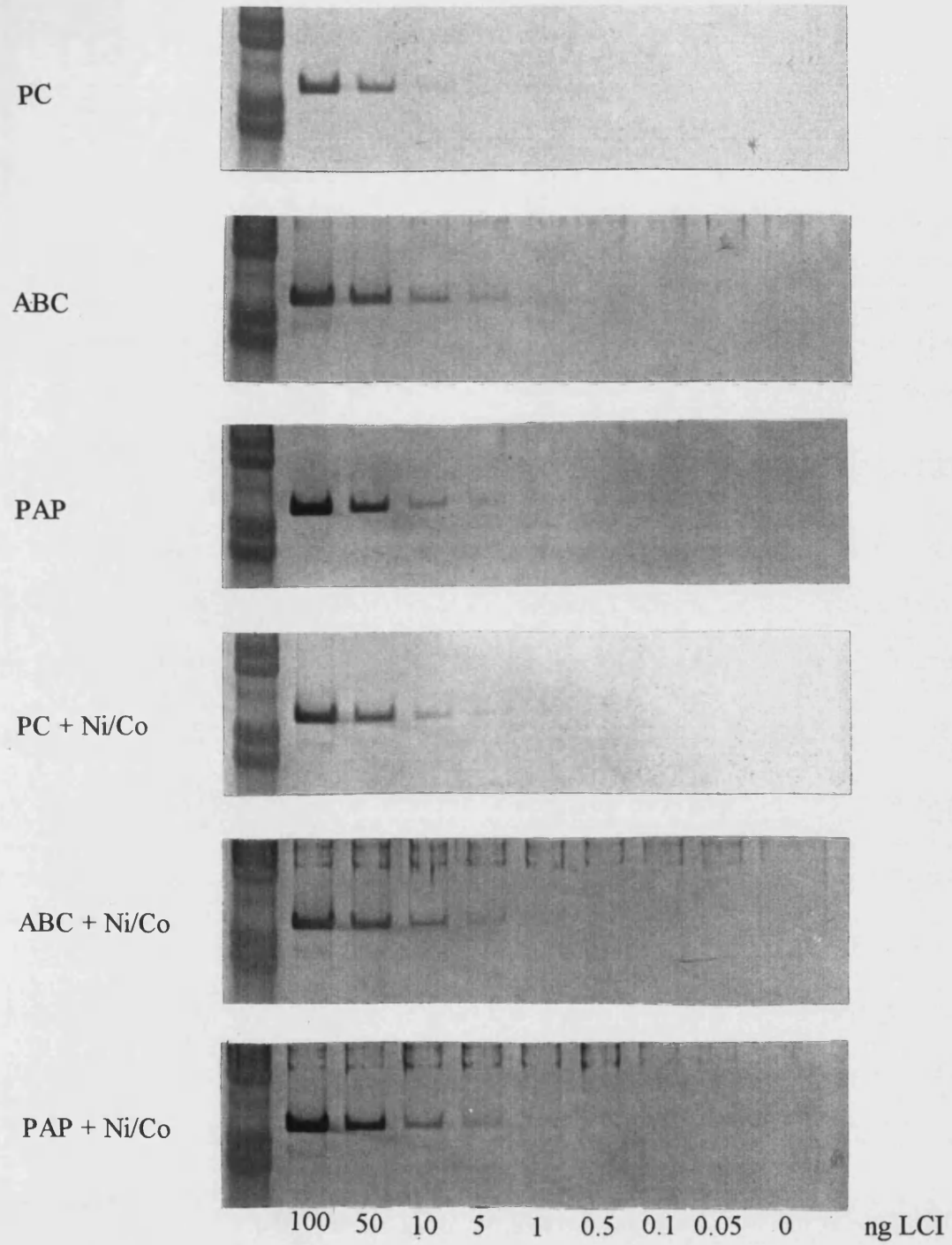


Figure 4. Comparison of immunoblotting detection methods
Six identical blots loaded with a range of concentrations of rh lipocortin I standard were developed using one or a combination of the following detection methods:
Peroxidase conjugate (PC)
Streptavidin-biotin complex (ABC)
Peroxidase-anti-peroxidase complex (PAP)
Nickel and Cobalt salt enhancement (Ni/Co)

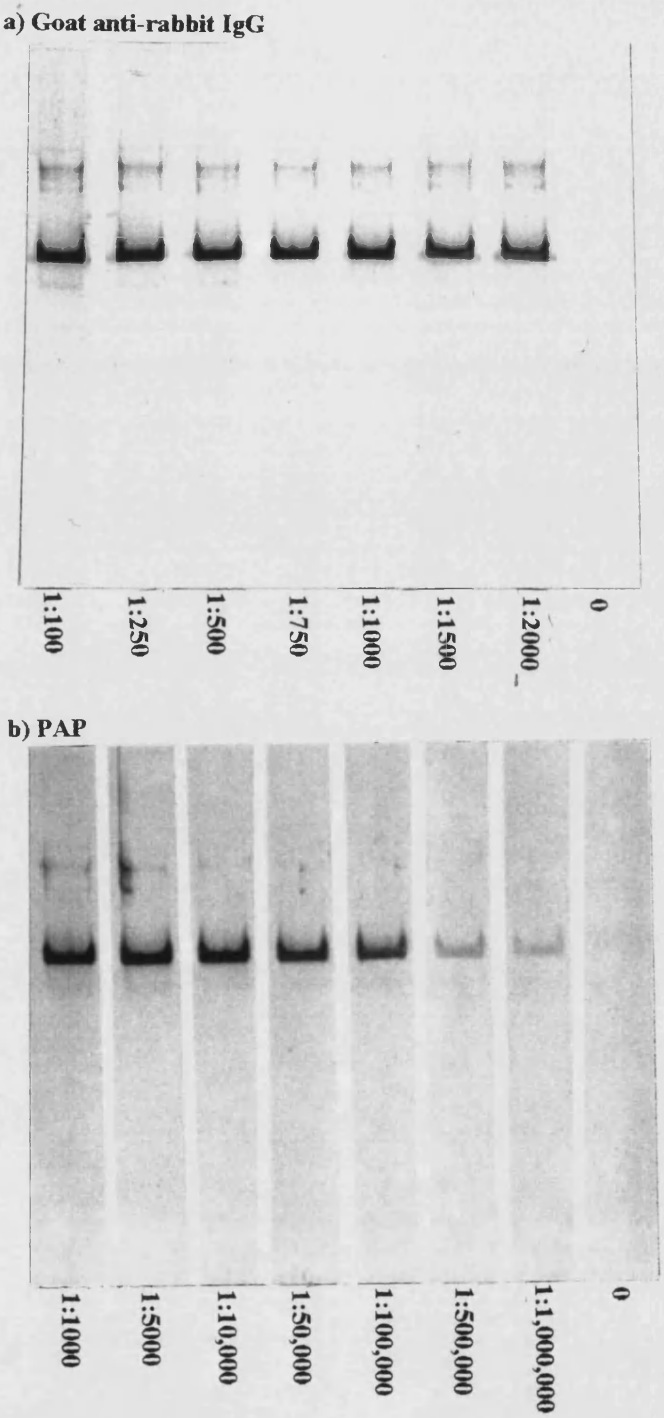


Figure 5. Titration of antibody reagents for the PAP immunoblotting detection method

a) Goat anti-rabbit IgG, optimum dilution: 1:500

b) PAP, optimum dilution: 1:10,000

(goat anti-rabbit IgG) and then developed in the normal way. The second blot was used to titrate the tertiary reagent (PAP complex) in the same manner. Optimum dilutions of goat anti-rabbit IgG and PAP were 1:500 and 1:10,000 respectively (Figure 5).

To check the specificity of staining, control blots containing representative CNS samples were subjected to the same immunoblotting procedure except that the primary antibody was omitted and replaced with PBS.

2.3.6 Estimation of Molecular Mass

To estimate the molecular mass of unknown bands on gels and blots, R_f values were calculated for standards and unknowns using the formula :

$$R_f \text{ (Ratio of fronts)} = \frac{\text{distance moved by band (mm)}}{\text{distance moved by dye front (mm)}}$$

The R_f values of standards were plotted against log molecular mass and the calibration curve obtained used to calculate the molecular mass of unknowns. Typical standard curves for low and high molecular mass markers are shown in Figures 6 and 7 respectively.

2.3.7 Densitometry of Immunoblots

In order to provide a semi-quantitative assessment of lipocortin levels, the intensity of immunoreactive bands was measured using a Joyce-Loebl Chromoscan 3 densitometer. Blots were scanned in reflectance mode (range: 0-1) using a KG3 (white light) filter, an aperture setting of 0.1 x 3 mm and a scan length of 60 mm. For background correction the valley-to-valley method was used with the pre-set ramp rate (10) and height, width and noise thresholds of 5, 4 and 3 respectively. Results were expressed as integral values.

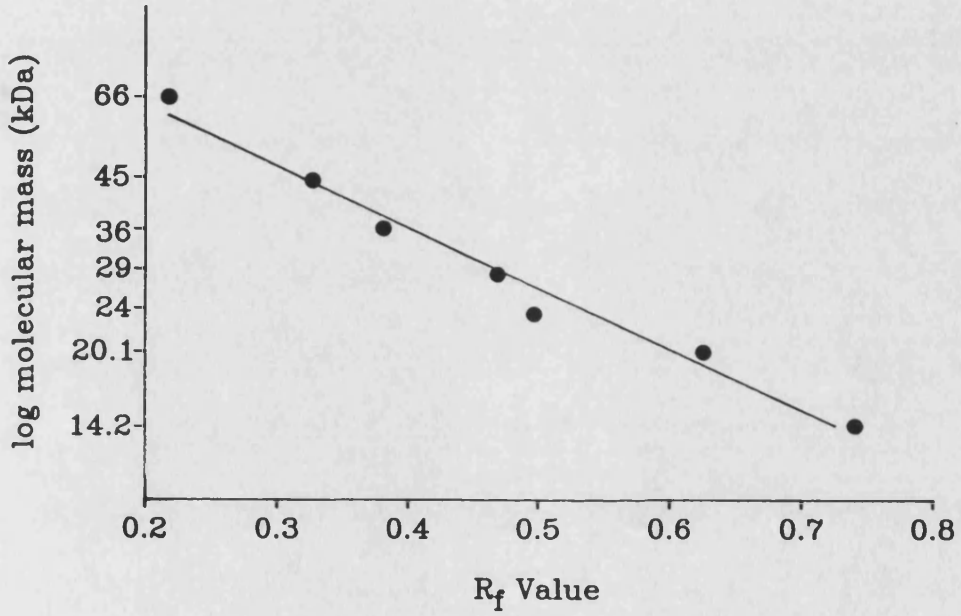


Figure 6. A typical standard curve obtained using SDS-PAGE low molecular mass markers

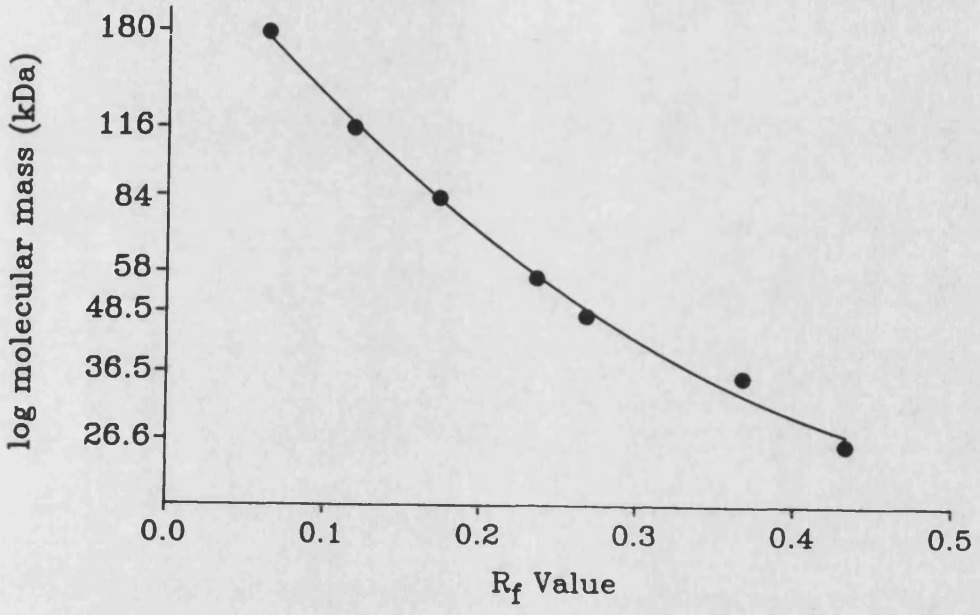
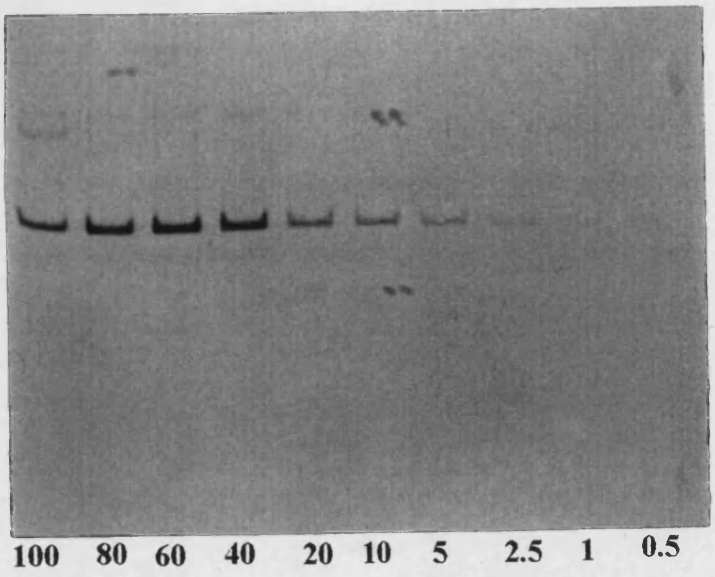


Figure 7. A representative standard curve obtained using pre-stained high molecular mass markers for SDS-PAGE and immunoblotting.
The electrophoretic mobility of protein standards is slightly altered by conjugation with the marker dye such that molecular masses calculated using pre-stained molecular mass markers were found to be accurate to within approximately $\pm$ 2 kDa.

a)



b)

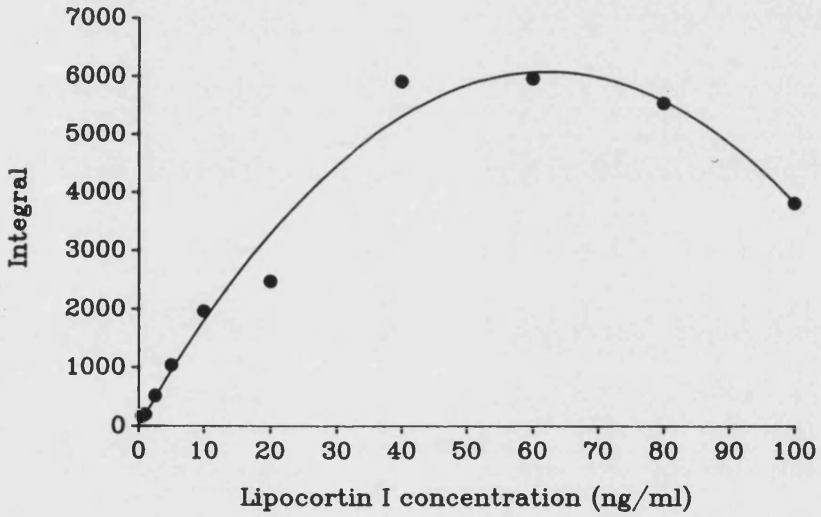


Figure 8. Densitometry of Western blots: relationship between protein concentration and integral value.
a) Blot loaded with a range of concentrations of lipocortin I
b) Plot of integral value against protein concentration

Linearity of the relationship between concentration and integral value was assessed by scanning a blot loaded with a range of concentrations of lipocortin I. A plot of concentration against integral value (Figure 8) showed that the relationship was reasonably linear over the range 1-40 ng. At higher concentrations integral values decreased from maximum. For this reason blots were always loaded with the minimum amount of protein sufficient to give a clear band falling within the linear range. In experiments on human CNS tissue, blot to blot variability was evaluated on blots probed for lipocortin I by calculating a coefficient of variation using integral values obtained by scanning the constant amount of lipocortin I standard (6.5 ng) which was run on all blots.

Statistical analysis of densitometry data from blots of human CNS tissue was performed by Dr P. Christie of Bath University Computer Services. To assess the significance of differences in the lipocortin content of various types of human CNS tissue, the integral values were first log transformed to stabilise the variance and then data for each protein was analysed by one way Analysis of Variance (ANOVA). For comparison of white matter subtypes three orthogonal contrasts were fitted: control versus all MS white matter subtypes, remote MS white matter versus plaque plus adjacent white matter, and adjacent white matter versus plaque. To determine whether differences between the groups might be explained by differing rates of breakdown of lipocortin I, a two way ANOVA was performed including putative breakdown products as a second factor.

2.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR LIPOCORTIN I

2.4.1 Preparation of Samples

2.4.1.1 Human CSF

EDTA was added to each CSF sample such that the final concentration was 1 mM. Samples were then freeze/thawed five times, clarified by centrifugation at 11,000 g for 5 min and the supernatants collected for assay.

2.4.1.2 Human CSF Supernatants

EDTA (1 mM) was also added to cell-free CSF supernatants.

2.4.1.3 Human CNS Tissue

Frozen supernatants from human CNS tissue homogenates that had previously been prepared for SDS-PAGE but which had not had sample buffer added, were thawed and adjusted to 1 mg/ml protein with homogenisation buffer.

To test for possible sources of interference, a range of dilutions of homogenisation medium and two representative CNS tissue samples (one rich in lipocortin, the other containing low levels of the protein) were spiked with a known amount of rh lipocortin I standard (10 or 20 ng). Controls were spiked with an equivalent volume of buffer.

2.4.1.4 Rat CNS Tissue

In one experiment rat CNS tissues were homogenised as described previously in homogenisation medium, with or without 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA, Sigma). After

freeze/thawing three times, samples were divided and one half centrifuged at 19,000 g for 30 min and the other at 100,000 g for 1 hr. Supernatants were collected, divided again, and one half of each was extracted three times with chloroform as follows: a double volume of chloroform was added, vortex mixed twice for 1 min, centrifuged at 220 g for 10 min to separate phases and the aqueous phase retained. Aliquots from each treatment were assayed spiked with a known amount of rh lipocortin I (20 ng) or with an equal volume of buffer.

2.4.2 ELISA

Following preparation as described above, CSF and CNS samples were assayed for lipocortin I using a "sandwich-type" ELISA (Smith *et al.* 1990). In this assay the wells of a microtitre plate are coated with monoclonal antibody to lipocortin I, remaining protein binding sites are blocked with foetal calf serum and samples and standards are then added. Lipocortin I binds specifically to the monoclonal antibody and polyclonal antisera to lipocortin I is then added which also binds to the lipocortin, thus forming an antibody-antigen-antibody "sandwich". The polyclonal antibody is then bound by a third enzyme conjugated antibody. Enzyme substrate is added to produce a colour reaction, the intensity of which is proportional to the amount of lipocortin present.

ELISA was performed in Nunc Maxisorp 96 well microtitre plates (Gibco). The reaction volume was always 100 µl and all incubations were for 1 hr at 37°C unless otherwise stated. For details of antibodies to lipocortin I see Table IV. The assay protocol is listed below:

1) All wells incubated overnight at 4°C with 2 µg/well purified mouse monoclonal antibody to lipocortin I (mAb 1B) in 0.05 M carbonate/bicarbonate buffer, pH 9.6
2) Washed x 3 in carbonate/bicarbonate buffer

3) Incubated 1% ($^{V}/_{V}$) foetal calf serum (Gibco) in PBS, pH 7.4 (Oxoid) (PBS-FCS)
4) Standards and samples were prepared and incubated as follows: Lipocortin I standard which had previously been diluted to 0.8 mg/ml in PBS-FCS was used to prepare doubling dilutions in PBS containing 1% Tween 20 (Sigma)(PBS-Tween), ranging from 4 µg-0.03 ng/well. Samples and standards were also diluted in PBS-Tween. Blanks contained PBS-Tween only.
5) Washed x 5 in 0.15 M sodium chloride solution containing 0.05% ($^{V}/_{V}$) Tween 20 (Saline-Tween)
6) Incubated with rabbit polyclonal antisera to lipocortin I (code: 842) diluted 1:1000 in PBS-Tween
7) Washed x 5 in Saline-Tween
8) Incubated with goat anti-rabbit alkaline phosphatase conjugate (Sigma) diluted 1:1000 in PBS-Tween
9) Washed x 5 in Saline-Tween
10) Enzyme substrate: 0.1% ($^{W}/_{V}$) disodium-*p*-nitrophenyl phosphate (Sigma) in carbonate/bicarbonate buffer added, plate covered in foil and incubated approximately 40 min at room temperature until colour developed
11) Optical density measured using a Titertek Multiscan MCC/340 at 405 nm with a 620 nm reference filter

A standard curve of optical density against log lipocortin I concentration was constructed and used to calculate the lipocortin content of unknown samples. A typical standard curve is shown in Figure 9.

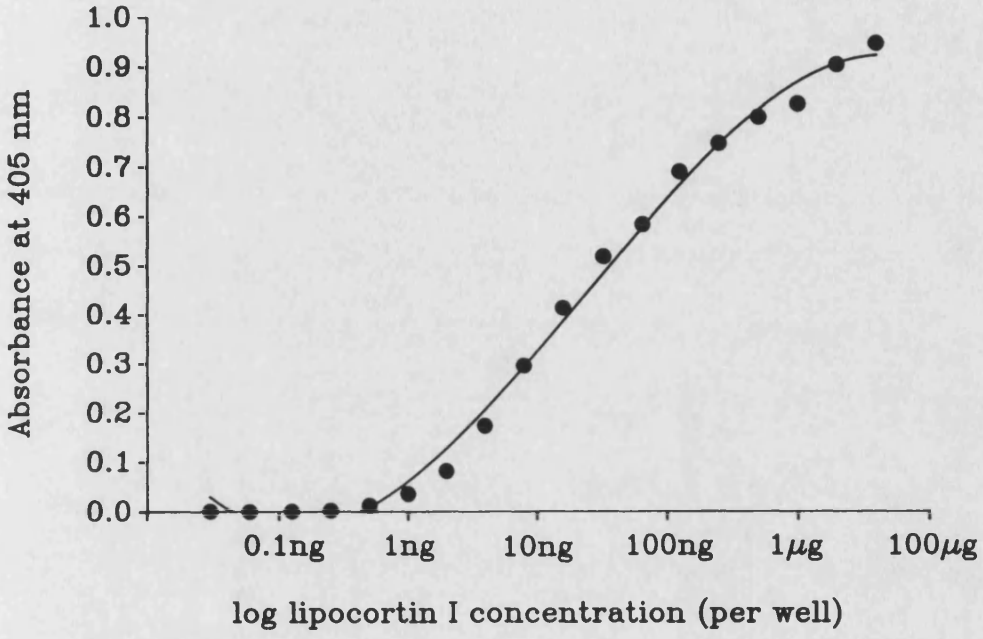


Figure 9. A typical standard curve obtained for the lipocortin I ELISA.

2.5 HISTOLOGY

2.5.1 Human CNS Tissue

Histology and immunohistochemistry were performed on human CNS tissue by Dr J. Newcombe, Institute of Neurology, London.

2.5.1.1 Histology

Sections 10 μm thick were cut from snap-frozen blocks of human CNS tissue using a cryostat and mounted onto glass slides. To assess cellular infiltration and the extent of demyelination, sections from each block were stained with H & E and Oil-Red-O using standard methods.

2.5.1.2 Immunohistochemistry for Lipocortin I

Immunohistochemistry was performed essentially as described by Newcombe and Cuzner (1988). Cryostat cut sections were mounted onto gelatin-coated slides and fixed in acetone for 10 min at 4°C. After incubation with the appropriate blocking sera, adjacent sections from each sample were probed either with rabbit antisera to lipocortin I (code: 842) or with a mouse monoclonal antibody directed against the astrocyte protein, glial fibrillary acidic protein (GFAP, Newcombe *et al.* 1986) or the pan macrophage marker EBM/11 (Dako). Sections were immunoperoxidase stained with a mouse or rabbit avidin-biotin complex kit (Vector Laboratories) using 0.05% ($^{w}/_{v}$) DAB (Sigma), 0.013% ($^{w}/_{v}$) H_2O_2 and 0.04% ($^{w}/_{v}$) nickel (II) chloride hexahydrate. Slides were then dehydrated, cleared in ethanol-xylene and mounted in DPX (BDH).

2.5.2 Rat CNS Tissue

2.5.2.1 Histology

In most experiments cervical spinal cords were fixed in Carnoy's fluid (Fava *et al.* 1989) comprising absolute alcohol, chloroform and acetic acid in the ratio 6:3:1 and then dehydrated and cleared as follows:

1) Carnoy's fluid	90 min
2) Absolute alcohol	1 hr, 3 changes
3) Cedar wood oil (Fisons)	1 hr, 2 changes

The cords were then stored in cedar wood oil prior to further processing.

Sectioning of the tissues and H & E staining were kindly performed by staff at the Department of Cellular Pathology, Royal United Hospital, Bath. Briefly, cedar wood oil was removed by overnight incubation with chloroform and the tissues were embedded in paraffin wax. Longitudinal horizontal sections, 6 μm thick, were cut at one standard depth which was defined as the widest point when starting from the uncut surface. Two sections from each block were mounted onto glass slides and stained with H & E. Sections for immunohistochemistry were mounted onto 3-aminopropyltriethoxysilane (Sigma) coated slides.

In one set of experiments in order to estimate CNS tissue lesion load, cervical spinal cords and cerebella were fixed in 10% formol saline, H & E stained sections were then prepared as described above. The number of perivascular infiltrates per section was determined by light microscopy.

2.5.2.2 Immunohistochemistry for Lipocortin I

Sections were de-paraffinized and brought through graded alcohols to water using the following protocol:

1) Xylene 2 min, 2 changes
2) 50% xylene in alcohol 30 sec
3) Absolute alcohol 30 sec
4) 95% alcohol 30 sec
5) 70% alcohol 30 sec
6) 40% alcohol 30 sec
7) Distilled water 30 sec

For the detection of immunoreactive lipocortin I a PAP method based on that used for Western blotting was employed. Slides were processed according to the following procedure and all incubations and washing steps were performed in PBS at room temperature unless otherwise stated:

1) Blocked with 0.5 ml ($^{V}/_{V}$) 10% goat serum (Gibco) for 30 min
2) Incubated with 20 µl antisera raised against rh lipocortin I (code: 842) under a glass coverslip in a humidified chamber, overnight at 4°C
3) Rinsed 3 x 5 min
4) Incubated 30 min with 0.5 ml goat anti-rabbit IgG (Sigma)
5) Rinsed 3 x 5 min
6) Incubated 30 min with 0.5 ml rabbit PAP (Sigma)
7) Rinsed 3 x 5 min
8) 0.05% ($^{W}/_{V}$) filtered DAB plus 0.03% ($^{W}/_{V}$) H_2O_2 until colour fully developed
9) Rinsed 3 x 5 min in distilled water

Antibody reagents 842, anti-rabbit IgG and PAP were titrated on serial sections taken from a typical EAE-diseased animal, optimum dilutions were found to be 1:250, 1:50 and 1:1,000 respectively (Figure 10).

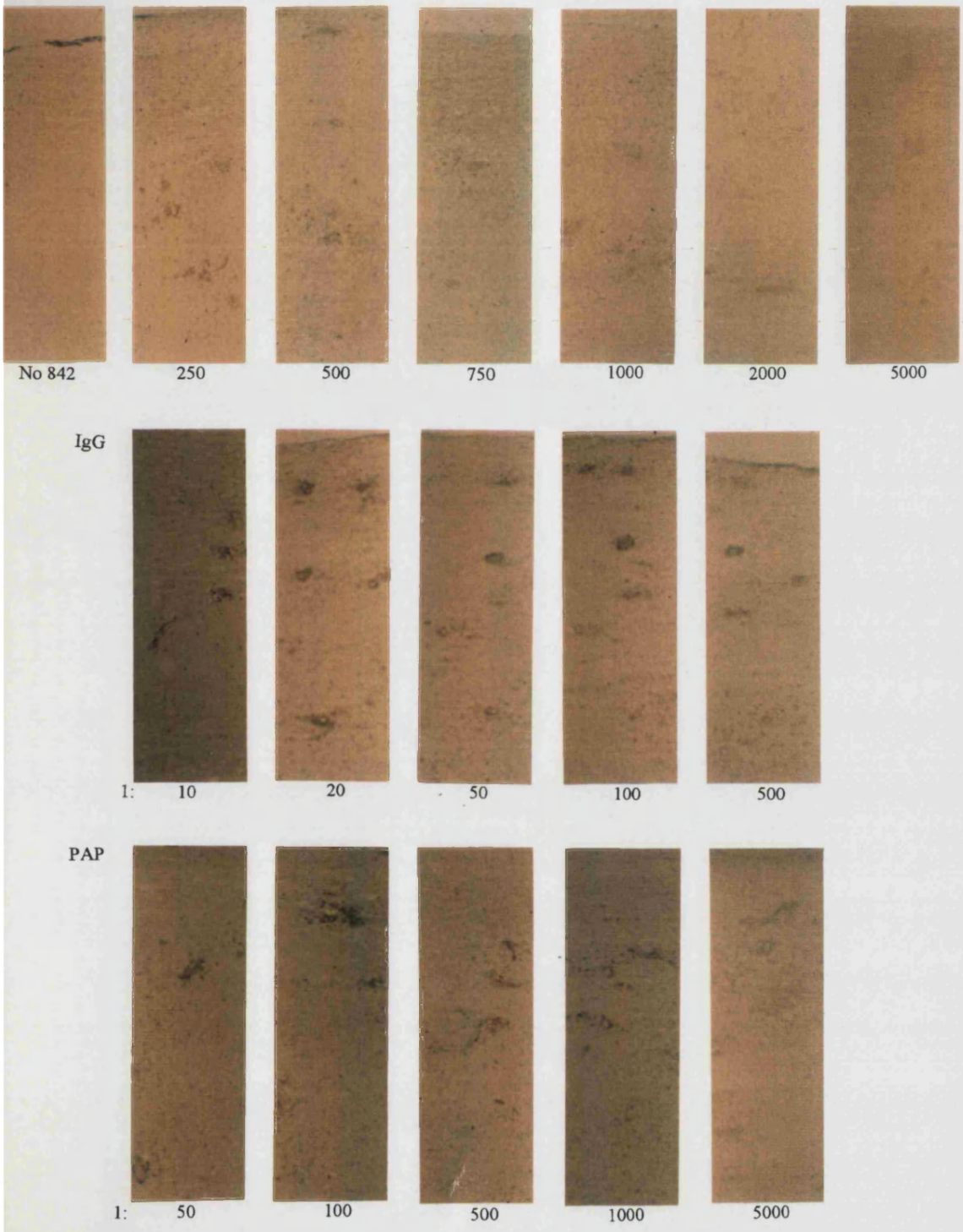


Figure 10. Titration of reagents for immunohistochemistry.
Antibody reagents for the peroxidase-anti-peroxidase (PAP) technique were titrated on serial sections of cervical spinal cord taken from a typical EAE-diseased animal. A representative area of each slide is shown at x40 magnification and the figures below each indicate the dilution of the reagent under test. Primary, secondary and tertiary reagents were titrated one at a time, in order, and the optimum dilutions of lipocortin I antisera (842), goat anti-rabbit IgG and PAP complex were found to be 1:250, 1:50 and 1:1000 respectively.

Control experiments were performed on sections taken from normal rats and from EAE-inoculated animals at the height of disease. To test for the presence of endogenous peroxidase activity and the effectiveness of a standard quenching technique (Polak and Van Noorden, 1986), slides were pre-incubated respectively with either PBS or 0.3% ($^{w}/_{v}$) H_2O_2 in PBS for 30 min at room temperature. The sections were then subjected to a sham immunohistochemistry procedure where all antibody reagents were replaced with PBS and then finally incubated with the DAB/H_2O_2 substrate. However no staining was detected on either quenched or non-quenched sections from normal or EAE-diseased animals (Figure 11) indicating that no endogenous peroxidase activity was present in the prepared sections.

Specificity of staining was determined by incubating slides with either PBS or non-immune rabbit serum (Sigma) instead of lipocortin antisera. In some experiments to further assess specificity of immunostaining, 842 was diluted 1:250 in PBS and aliquots incubated with a range of concentrations of rh lipocortin I (0.1-100 µg/ml), firstly for 1 hr at 37°C and then overnight at 4°C. This pre-adsorbed antisera was then used in place of 842 after which the slides were processed in the normal way.

Following immunohistochemistry, all sections were lightly counterstained with toluidine blue:

1) 1% ($^{w}/_{v}$) aqueous toluidine blue	1-4 hr
2) Rinsed distilled water	2 sec
3) Differentiated in 95% alcohol	2 sec
4) Dehydrated in absolute alcohol	30 sec, 3 changes
5) 50% xylene in alcohol	30 sec
6) Cleared in xylene	30 sec, 2 changes

7) Blotted dry with filter paper and mounted in DPX (BDH)

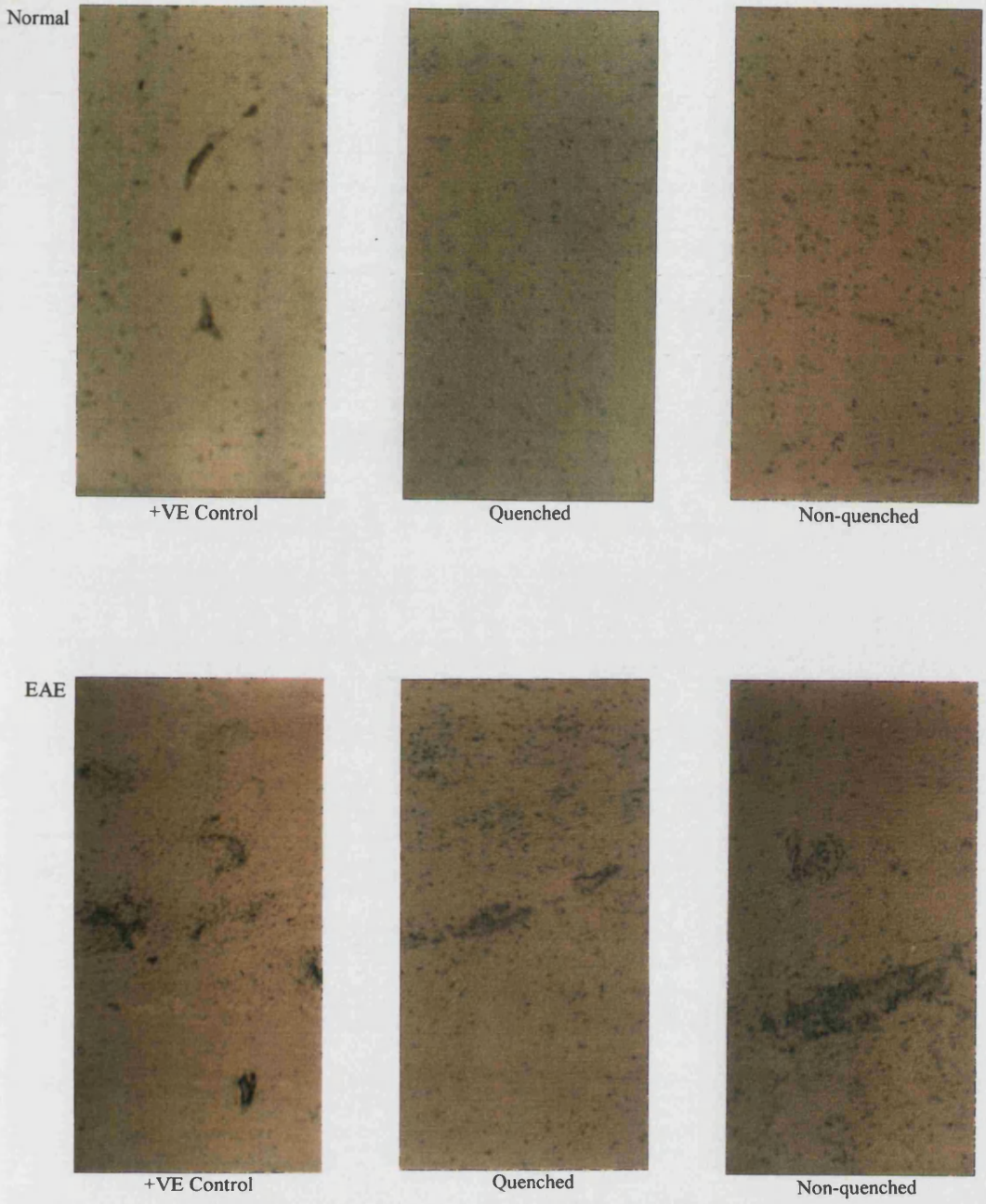


Figure 11. Control sections: test for endogenous peroxidase
Cervical spinal cord sections from normal and EAE-diseased rats were incubated with 0.3% H_2O_2 to quench endogenous peroxidase (Quenched). Control sections were incubated with PBS (Non-quenched). Sections were then subjected to a sham immunohistochemistry procedure, where all antibody reagents were replaced with PBS and finally, incubated with DAB/H_2O_2 substrate to test for endogenous peroxidase. +VE controls were developed in the normal way. Magnification x40.

Slides were observed and photographed using a Nikon Optiphot-2 microscope and Kodak Vericolor III professional film.

2.6 RADIOIMMUNOASSAY FOR CORTICOSTERONE

Corticosterone in rat serum was measured using an ICN RSL ^{125}I-corticosterone radioimmunoassay (RIA) kit obtained from IDS. The basis of this assay is that the corticosterone present in an unknown serum sample competes with a known quantity of ^{125}I-labelled corticosterone to bind a limited amount of specific antibody. Thus the more corticosterone present in the sample, the less radiolabelled corticosterone is able to bind to the antibody. The antigen-antibody complexes formed are separated from unbound corticosterone by precipitation and centrifugation and the amount of radiolabelled corticosterone complexed with antibody is estimated by gamma-counting the pellet. The radioactive counts are used to calculate the percentage of ^{125}I-corticosterone bound, which is inversely proportional to the amount of corticosterone in the sample.

The RIA has a range of 25-1,000 ng/ml and was performed according to the manufacturer's instructions, except that serum samples which were below the limit of detection were assayed at 1:50 dilution instead of the recommended 1:200. To reduce the influence of assay to assay variation, whenever possible serum samples from the same experiment were run in the same RIA.

2.6.1 Assay Protocol

The assay was performed in duplicate in 12 x 75 mm borosilicate disposable glass tubes (Kemble).

1) Rat serum thawed and diluted 1:200 (or 1:50) with steroid diluent

2) 0.3 ml steroid diluent added to non-specific binding (NSB) tubes and 0.1 ml to zero calibrator tubes
3) 0.1 ml of corticosterone standards, rat serum controls or diluted rat serum samples added to appropriate tubes
4) 0.2 ml ^{125}I-corticosterone added to all tubes
5) 0.2 ml anti-corticosterone added to all tubes except NSB
6) Tubes vortexed and incubated for 2 hr at room temperature
7) 0.5 ml precipitant solution added to all tubes and vortexed thoroughly
8) All tubes centrifuged at 1,000 g for 15 min and then the supernatant decanted and the rim of the tube blotted dry on absorbent paper
9) Precipitate counted in a LKB 1277 Gammamaster automatic gamma counter for 2 min

2.6.2 Calculations and Statistics

Results were expressed in counts per minute (CPM) and the percentage ^{125}I-corticosterone bound was calculated for both standards and samples using the formula:

$$\%\ B/Bo = \frac{\bar{x}\ CPM\ (sample) - \bar{x}\ CPM\ (NSB)}{\bar{x}\ CPM\ (0\ ng/ml) - \bar{x}\ CPM\ (NSB)} \times 100$$

Where: $\%\ B/Bo$ = percentage ^{125}I-corticosterone bound
$\bar{x}$ CPM (sample) = mean CPM of standard or sample
$\bar{x}$ CPM (NSB) = mean CPM of non-specific binding
$\bar{x}$ CPM (0 ng/ml) = mean CPM of standard containing 0 ng/ml corticosterone i.e. 100% binding tube

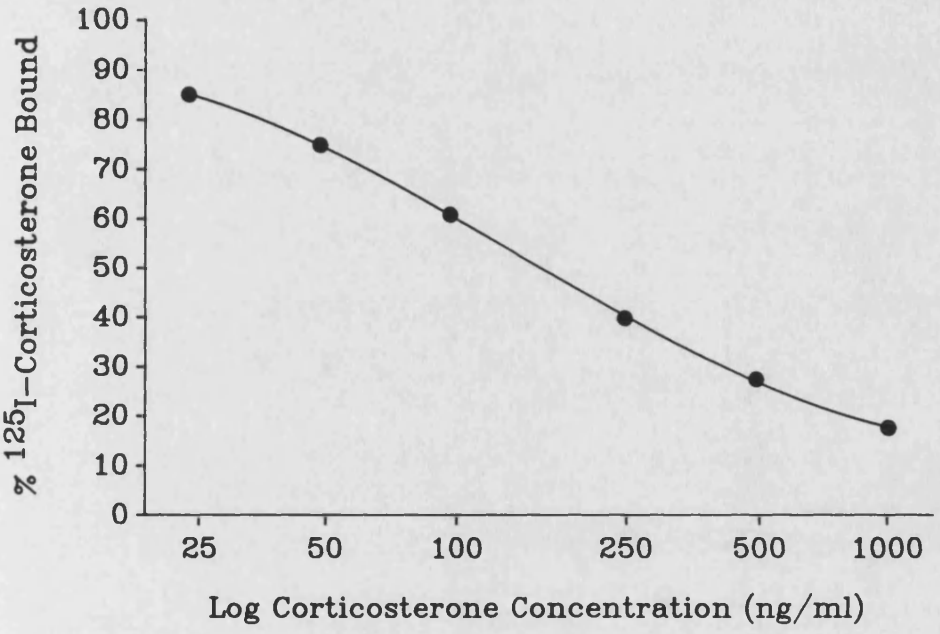


Figure 12. A representative standard curve obtained using the ICN ^{125}I-corticosterone radioimmunoassay kit

A standard curve of percentage bound against log concentration was constructed and the corticosterone concentration of unknown samples read directly from the curve. A typical standard curve is shown in Figure 12. Values obtained for samples which had been diluted 1:50 were corrected by dividing by four.

Simple statistical comparisons between two different groups were performed using the Mann-Whitney U test, with the Bonferroni Correction when three groups were compared. The Wilcoxon Signed-Rank test was used to analyse the significance of differences between samples collected from the same group before and after treatment. For experiments requiring multiple comparisons between several groups, results were assessed using one way ANOVA and the significance of differences between group means determined using the Newman-Keuls procedure (Snedecor and Cochran, 1967).

RESULTS

PART A: LIPOCORTINS IN THE CNS OF MS PATIENTS AND CONTROLS

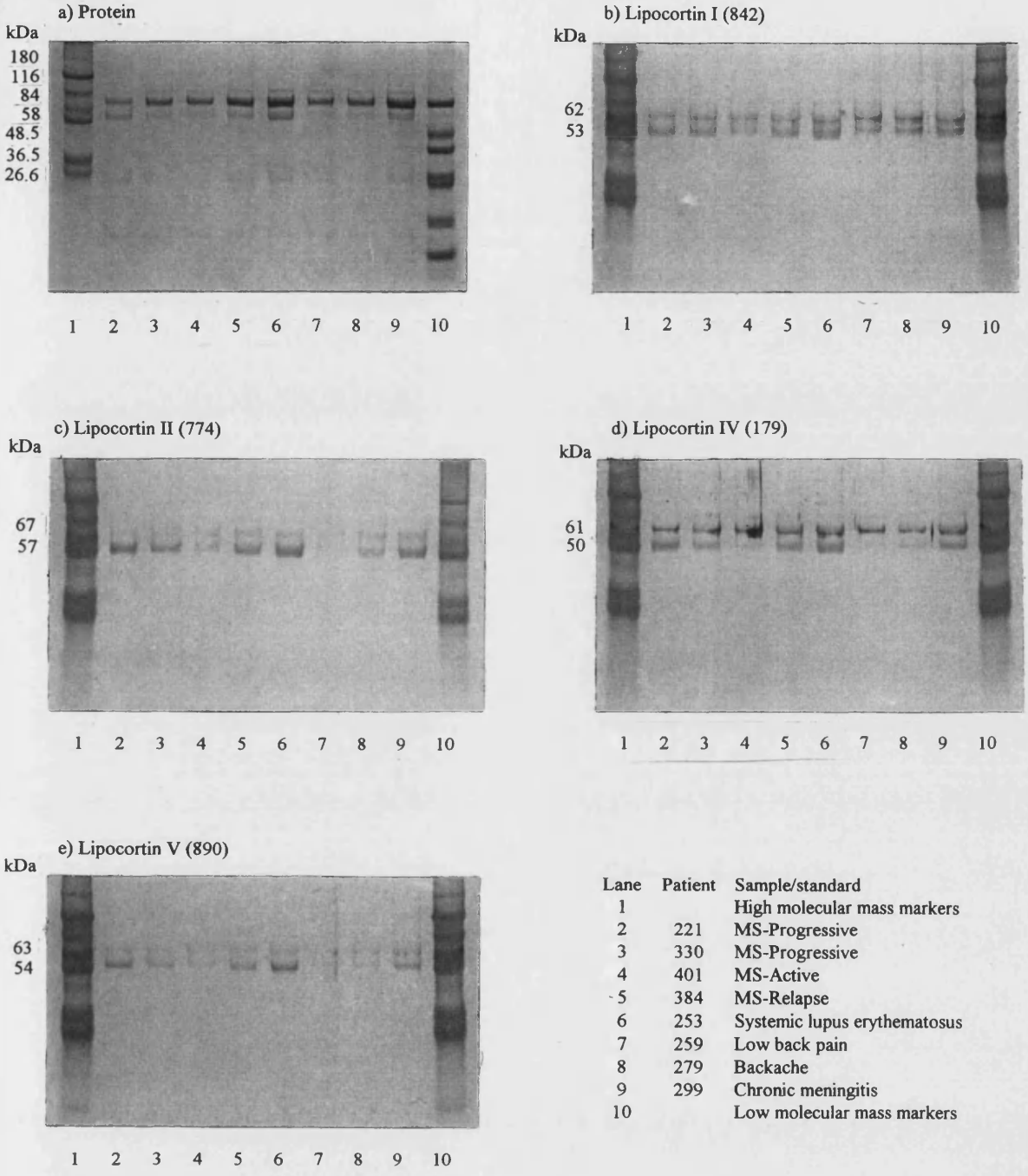
3.1 CEREBROSPINAL FLUID

3.1.1 Cell-free Supernatants

Cell-free CSF supernatants from patients with MS and control cases of systemic lupus erythematosus, chronic meningitis and idiopathic back pain were assessed for the presence of lipocortins I, II, IV and V by SDS-PAGE and Western blotting using the peroxidase conjugate technique. A gel stained for total protein with PAGE Blue 83, confirmed that adequate separation of CSF proteins could be achieved using this method and showed that the samples contained small but variable amounts of protein (Figure 13a). However, when Western blots of these samples were probed for lipocortins I, II, IV and V, no immunoreactive bands of appropriate molecular mass were detected in either MS or control CSFs, although very faint bands between 50 and 70 kDa were observed in all lanes of each blot (Figure 13 b-e).

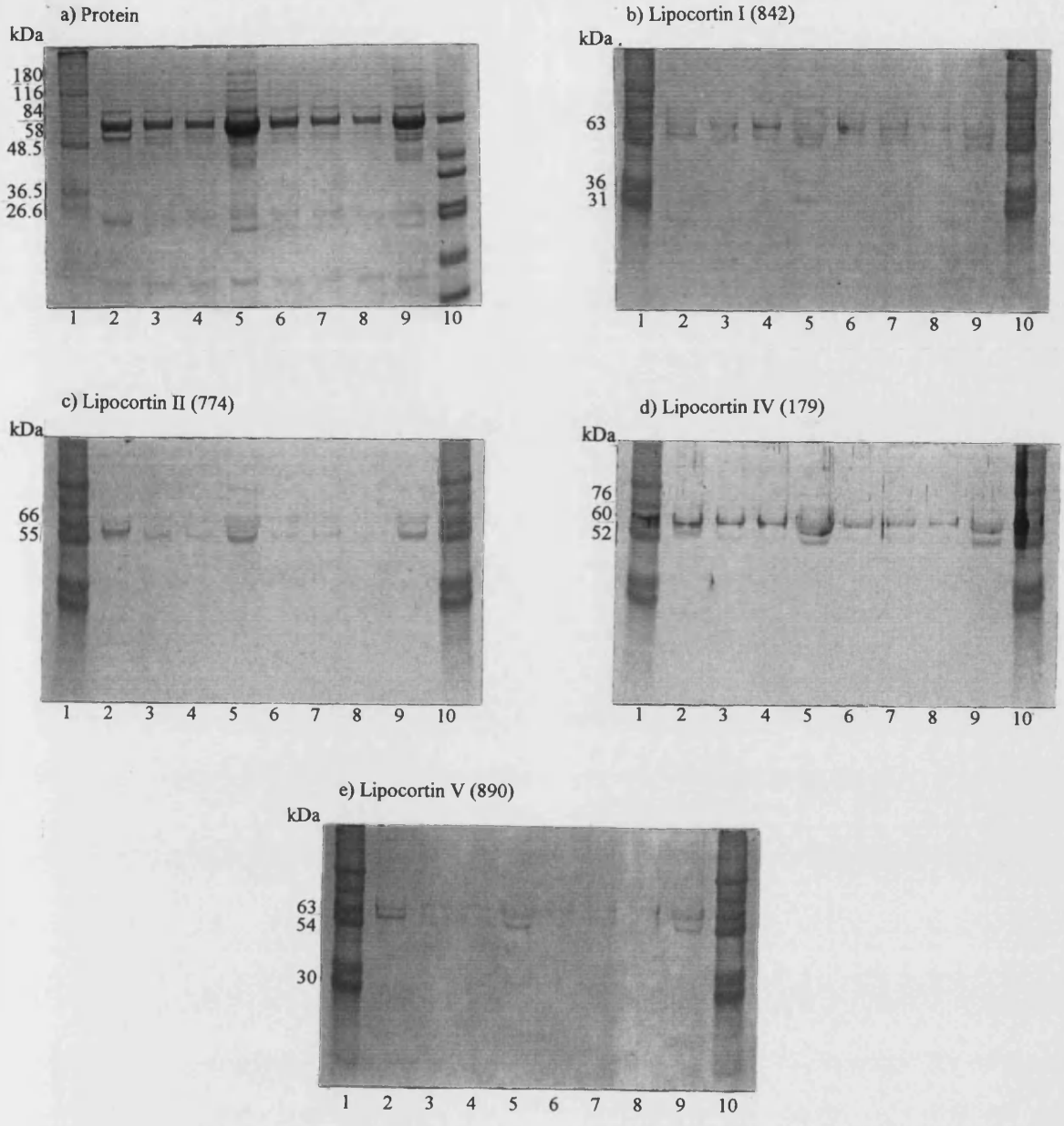
3.1.2 Whole CSF

Since lipocortins were undetectable in any of the cell-free supernatants studied, the same technique was used to investigate the lipocortin content of whole CSF which was prepared by repeated freezing and thawing in order to lyse the cells and thus allow measurement of both intracellular and extracellular protein. Samples were obtained from patients with a variety of diseases although only one of these was diagnosed as having MS. A protein-stained gel and Western blots from one representative experiment are shown in Figure 14. A summary of all the results from this set of experiments is shown in Table V which also details the CSF



Lane	Patient	Sample/standard
1		High molecular mass markers
2	221	MS-Progressive
3	330	MS-Progressive
4	401	MS-Active
5	384	MS-Relapse
6	253	Systemic lupus erythematosus
7	259	Low back pain
8	279	Backache
9	299	Chronic meningitis
10		Low molecular mass markers

Figure 13. Immunoblots for lipocortins in cell-free CSF from MS patients and controls
(a) Protein-stained gel and (b-e) Western blots of cell-free CSF supernatants from MS patients and controls probed for lipocortins I, II, IV and V. Code numbers of antibodies are shown in parenthesis.



Lane	Patient/ Sample	Diagnosis	Protein g/l	Leukocytes /mm^3	Erythrocytes /mm^3
1	MWM				
2	AW	Multiple myeloma	0.87	<1	570
3	YC	-	0.28	<1	3
4	CT	Malignant lymphoma	<0.3	<1	115
5	DT	Astrocytoma	2.9	90	10
6	EN	Dementia	-	-	-
7	PH	Viral infection	<0.3	<1	<1
8	DM	Migraine	-	-	-
9	EP	Tuberculous meningitis	1.14	162	10
10	MWM				

Figure 14. Lipocortins in whole human CSF
(a) Protein-stained gel and (b-e) Western blots of whole human CSF lysate from patients with a variety of diseases probed for lipocortins I, II, IV and V. Code numbers of antibodies are shown in parenthesis. MWM, molecular mass markers.

Table V

Lipocortins in human CSF lysate

Patient	Diagnosis	CSF Protein g/l	CSF Leukocytes /mm^3	CSF Erythrocytes /mm^3	Lipocortins I	II	IV	V
AW	Multiple myeloma	0.87	<1	570	–	–	–	–
YC	–	0.28	<1	3	–	–	–	–
CT	Malignant lymphoma	<0.3	<1	115	–	–	–	–
DT	Astrocytoma	2.9	90	10	+	–	–	+
EN	Dementia	–	–	–	–	–	–	–
PH	Viral Infection	<0.3	<1	<1	–	–	–	–
DM	Migraine	–	–	–	–	–	–	–
EP	Tuberc. meningitis	1.14	162	10	?	–	–	–
EP	Tuberc. meningitis	–	–	–	?	–	–	–
KD	Vit A deficiency	1.45	1	10	–	–	–	–
LS	Convulsions	–	–	–	–	–	–	–
DT	Astrocytoma	2.7	50	5800	+	–	–	–
SR	Viral infection	<0.3	<1	<1	–	–	–	–
EA	Mononeuritis	0.84	<1	2	–	–	–	–
BJ	–	0.34	2	505	–	–	–	–
KN	Deform of ankle & foot	0.50	<1	4	–	–	–	–
HC	Pylonephritis	<0.3	1	6	–	–	–	–
GW	Urinary tract infection	<0.3	<1	<1	–	–	–	–
LN	Multiple sclerosis	0.68	3	<1	–	–	–	–
TC	Pyrexia	<0.3	<1	<1	–	–	–	–
SW	Backache	0.39	<1	<1	–	–	–	–
NW	–	0.7	<1	175	–	–	–	–
JB	Backache	0.76	<1	1120	–	–	–	–
LS	Slipped disc	1.38	<1	<1	–	–	–	–
RJ	Upper resp tract infect	<0.3	<1	<1	–	–	–	–
SM	Peripheral neuropathy	<0.3	<1	<1	–	–	–	–
KG	–	3.72	3320	280	+	–	–	+
CB	Migraine	<0.3	<1	15	–	–	–	–
LT	–	1.3	<1	<1	–	–	–	–
MP	–	<0.3	<1	<1	–	–	–	–
MB	Stroke	1.0	6	3	–	–	–	–
IG	Headache	<0.3	<1	4	–	–	–	–
AR	Chest infection	<0.3	<1	<1	–	–	–	–
MH	Backache	<0.3	<1	205	–	–	–	–
DC	Breast cancer	0.5	<1	13	–	–	–	–
ET	Polyneuritis	0.56	<1	<1	–	–	–	–
CM	–	–	–	–	–	–	–	–
CS	Ear infection	<0.3	<1	325	–	–	–	–
JB	Meningococcal meningitis	4.68	1240	150	+	–	–	–

Lipocortins were detected by Western blotting using the following polyclonal antibodies: lipocortin I, 842; lipocortin II, 774; lipocortin IV, 179; lipocortin V, 890. +, lipocortin present; -, lipocortin not detected; ? indicates presence of lipocortin immunoreactive protein of lower molecular mass (31 kDa).

protein concentration, erythrocyte and leukocyte counts plus the final diagnosis, where available.

On blots probed for lipocortin I low levels of an approximately 36 kDa immunoreactive protein were detected in four out of the 39 samples tested. The molecular mass of this band is very similar to that reported for human lipocortin I (37 kDa) by Wallner *et al.* (1986). Of the four samples containing lipocortin I-like protein, two were diagnosed as astrocytomas, one as meningitis and one was of unknown pathology. In addition, a lipocortin I-immunoreactive protein of a slightly lower molecular mass (approximately 31 kDa) was observed in two samples from another patient with meningitis. It is likely that this band is a breakdown product of lipocortin I (Pepinsky *et al.* 1986). In each case the presence of lipocortin I immunoreactivity was associated with substantially elevated CSF protein levels and a very high leukocyte count.

Two of the samples in which lipocortin I-like protein was detected, also exhibited very low levels of a 30 kDa band on blots probed for lipocortin V. The molecular mass of this band is close to that reported by Comera *et al.* (1989) (32 kDa) for lipocortin V isolated from human peripheral blood mononuclear cells. Lipocortins II and IV did not appear to be present in any of the samples tested. Several very faint higher molecular mass bands between 50 and 70 kDa were observed on all blots.

3.1.3 ELISA for Lipocortin I in CSF

Selected CSF samples were assayed for lipocortin I using the more sensitive ELISA technique, which was performed by Dr N. Goulding. This confirmed the presence of nanogram amounts of the protein in two samples of whole CSF which had previously been shown to contain low levels of the protein by Western blotting. Furthermore, ELISA of cell-free CSF supernatants which had

Table VI

Quantitation of lipocortin I in human CSF by ELISA

Patient	Diagnosis	CSF Type	Lipocortin I ng/ml
401	MS-Active	Cell-free Supernatant	-
384	MS-Relapse	"	-
457	MS-Relapse	"	-
221	MS-Progressive	"	0.8
330	MS-Progressive	"	0.8
471	MS-Chronic progressive	"	-
299	Chronic meningitis	"	3.29
253	Systemic lupus erythematosus	"	0.8
259	Low back pain	"	-
279	Backache	"	-
DT	Astrocytoma	Lysate	15.8
EP	Tuberculous meningitis	"	7.8

Detection limit of ELISA is approximately 0.8 ng/ml. (-) indicates lipocortin I concentration was below limit of detection.

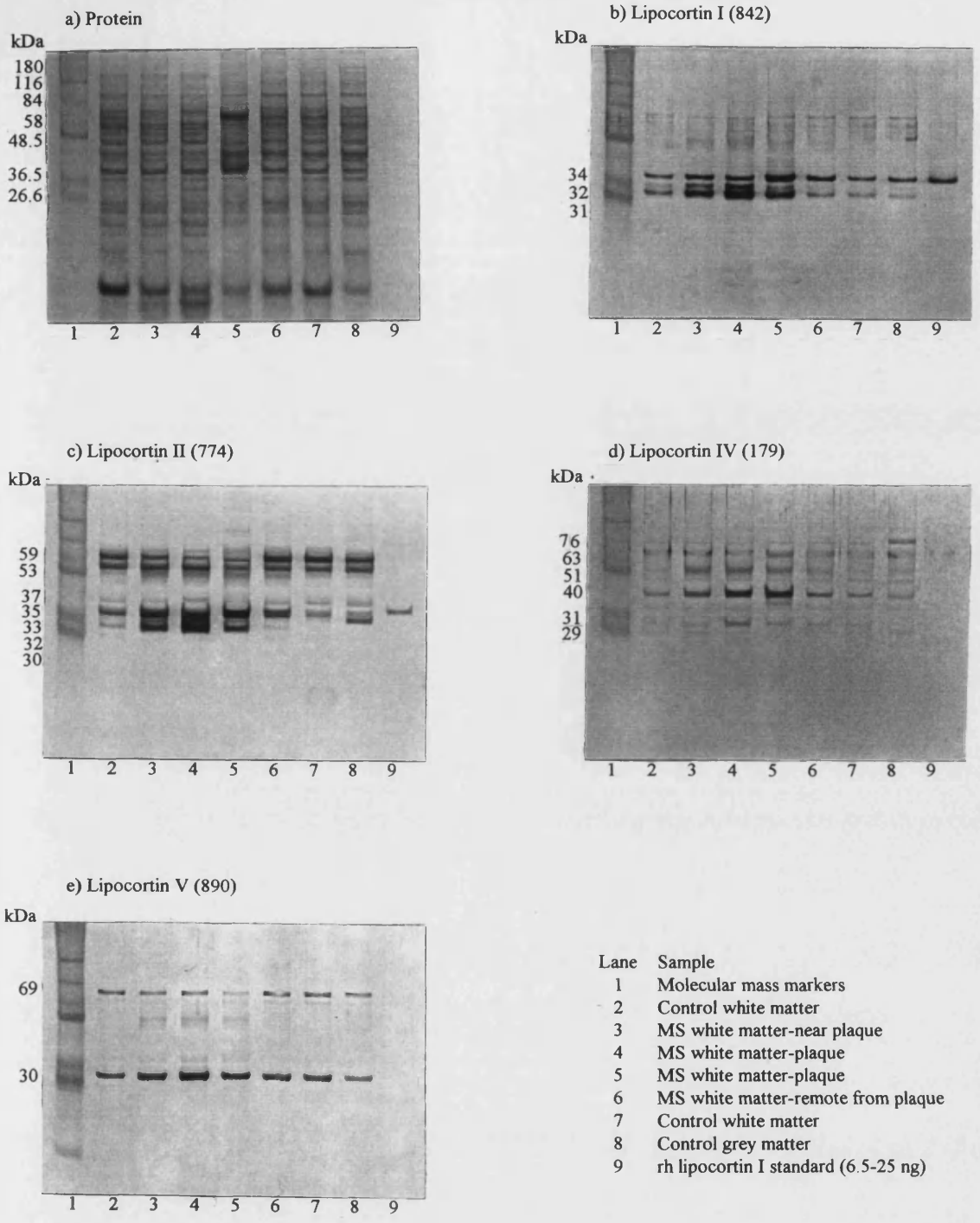
appeared negative for lipocortin I using Western blotting, revealed the presence of extremely low levels of the protein in several samples including two from patients with progressive MS (Table VI).

3.2 CNS TISSUE

3.2.1 Detection of Lipocortins by SDS-PAGE and Immunoblotting

As only limited amounts of lipocortin were detected in CSF and that which was present appeared to be cell-associated, attention was turned to CNS tissue. Using SDS-PAGE and the PAP method lipocortins I, II, IV and V were assessed in samples of white and grey matter from normal controls, and in plaque tissue, white matter adjacent to a plaque, white matter remote from a plaque and grey matter from patients with MS. The relative amounts of lipocortin in each sample-type were then semi-quantitatively estimated by densitometry. The results of this work have been published in the Journal of Neuroimmunology (Elderfield *et al.* 1992).

In a preliminary experiment to determine the optimum loading of the gels for good visualisation, adequate separation of bands and accurate densitometry, 14 samples were run at several different protein concentrations namely: 20, 10 and 5 μg total protein per lane. In each case the same qualitative differences were observed; 5 μg per lane was found to be optimum for lipocortins I and V and 10 μg and 20 μg for lipocortins IV and II respectively. Results obtained in one typical experiment are shown in Figure 15. Figure 15a shows a gel stained for total protein and indicates that adequate separation of proteins occurred and that following adjustment of protein concentration all samples contained equivalent amounts of protein. Figure 15b-e shows Western blots probed for lipocortins I, II, IV and V respectively.



Lane	Sample
1	Molecular mass markers
2	Control white matter
3	MS white matter-near plaque
4	MS white matter-plaque
5	MS white matter-plaque
6	MS white matter-remote from plaque
7	Control white matter
8	Control grey matter
9	rh lipocortin I standard (6.5-25 ng)

Figure 15. Lipocortins in CNS tissues from MS patients and controls
(a) Protein stained-gel and (b-e) Western blots of CNS tissues from MS patients and controls probed for lipocortins I, II, IV and V. Code numbers of antibodies are shown in parenthesis.

On blots probed for lipocortin I using antibody 842, three distinct immunoreactive bands of approximately 37, 35 and 33 kDa were consistently detected in almost all samples. The 37 kDa band co-migrated with rh lipocortin I standard and the molecular mass of this band is concordant with that reported for human lipocortin I by Wallner *et al.* (1986). The two lower molecular mass species may represent breakdown products of lipocortin I, particularly the 33 kDa band which migrated to the same position as a small amount of contaminant protein observed in the lipocortin I standard which was originally a highly purified recombinant protein preparation (Wallner *et al.* 1986). Similar lower molecular mass species have been observed in human tissues by other workers and appear to be due to enzymatic degradation of lipocortin I (Huang *et al.* 1987, Smith *et al.* 1990b, Ambrose & Hunninghake 1990a). In order to assess possible differences between the groups in the degree of lipocortin I degradation, following densitometry a preliminary Two Way Analysis of Variance was performed including the three bands as a second factor, this showed no difference between any of the groups in the proportion of protein in each band. An alternative explanation for the presence of the 35 kDa band is the possibility that this may be lipocortin III, as it is of the appropriate molecular mass and the 842 antibody is known to exhibit strong cross-reactivity with this protein (Pepinsky *et al.* 1988). Many bands between 40 and 80 kDa were observed on these blots including one at 68 kDa, however they were very faint compared to the main bands.

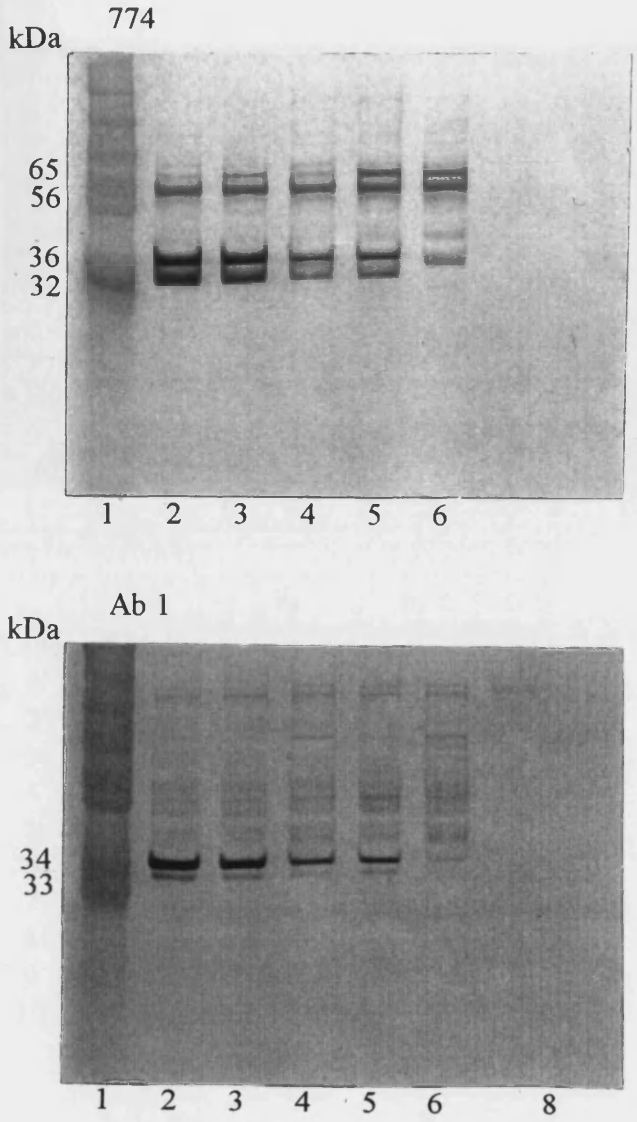
On blots probed for lipocortin II with antibody 774, several bands between 30 and 40 kDa were observed, although the major band was approximately 35 kDa which corresponds to the reported molecular mass of lipocortin II (Huang *et al.* 1986, Pepinsky *et al.* 1988). In addition, two higher molecular mass bands were observed at approximately 61 and 54 kDa and the antibody was also found to cross-react with lipocortin I standard. To check the identity of the major band

some samples were re-assayed using a different antibody raised against rh lipocortin II (Ab 1). This antibody, which did not cross-react with lipocortin I, gave only two bands in the 30-40 kDa region, the main band having a molecular mass of approximately 34 kDa. Quantitative changes in lipocortin II-like protein appeared to be identical with both antibodies (Figure 16).

Several bands were present on blots probed for lipocortin IV (antibody 179), the predominant species having a molecular mass of approximately 40 kDa. Blots probed for lipocortin V (antibody 890) exhibited two main bands one at approximately 30 kDa, which is close to the molecular mass reported by Comera *et al.* (1989) for lipocortin V (32 kDa), and another at approximately 67 kDa. Neither antiserum appeared to cross-react with lipocortin I.

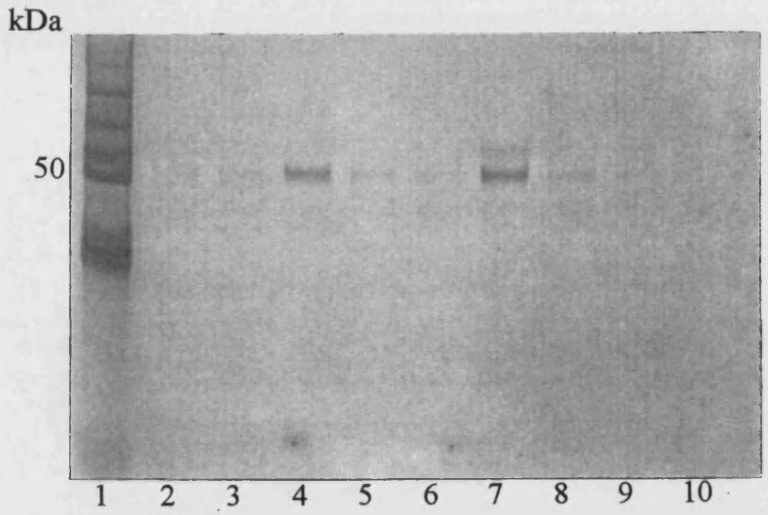
Figure 17 shows a control blot comprising eight typical samples, where the primary antibody was omitted to determine non-specific staining. Only one very faint non-specific band was observed at approximately 50 kDa in each sample indicating that all other bands are due to binding of lipocortin antisera.

In total 35 CNS tissue samples were assayed with 5-7 samples per group. For purposes of comparison, three representative samples from each group are shown in Figure 18. On all blots the molecular mass of the major band was close to that reported for the appropriate lipocortin in the literature. The intensity of the main band in each sample was therefore measured using densitometry in order to provide a semi-quantitative assessment of the amount of each lipocortin-like protein. Densitometry data for all samples are summarised in Table VII. To reduce the influence of blot-to-blot variations in staining intensity, samples in the same group were randomised between different blots. Using the densitometer values obtained by scanning the constant amount (6.5 ng) of rh lipocortin I run on all blots probed for this protein, a coefficient of variation of 19.6 % was obtained.



Lane	Sample
1	High molecular mass markers
2	MS white matter-plaque
3	MS white matter-near plaque
4	MS grey matter
5	MS white matter-remote from plaque
6	Control grey matter
8	rh lipocortin I (25 ng)

Figure 16. Comparison of lipocortin II antisera
Western blots containing identical CNS tissue samples from MS patients and controls probed with two different lipocortin II antisera, 774 and Ab 1.



Lane	Sample
1	High molecular mass markers
2	Control white matter
3	Control grey matter
4	MS white matter-remote from plaque
5	MS grey matter
6	Control white matter
7	MS white matter-remote from plaque
8	MS grey matter
9	Control white matter
10	rh lipocortin I (25 ng)

Figure 17. Control blot omitting primary antiserum
Western blot of human CNS tissues from MS patients and controls immunoblotted using the PAP method but incubated with PBS in place of specific lipocortin antiserum.

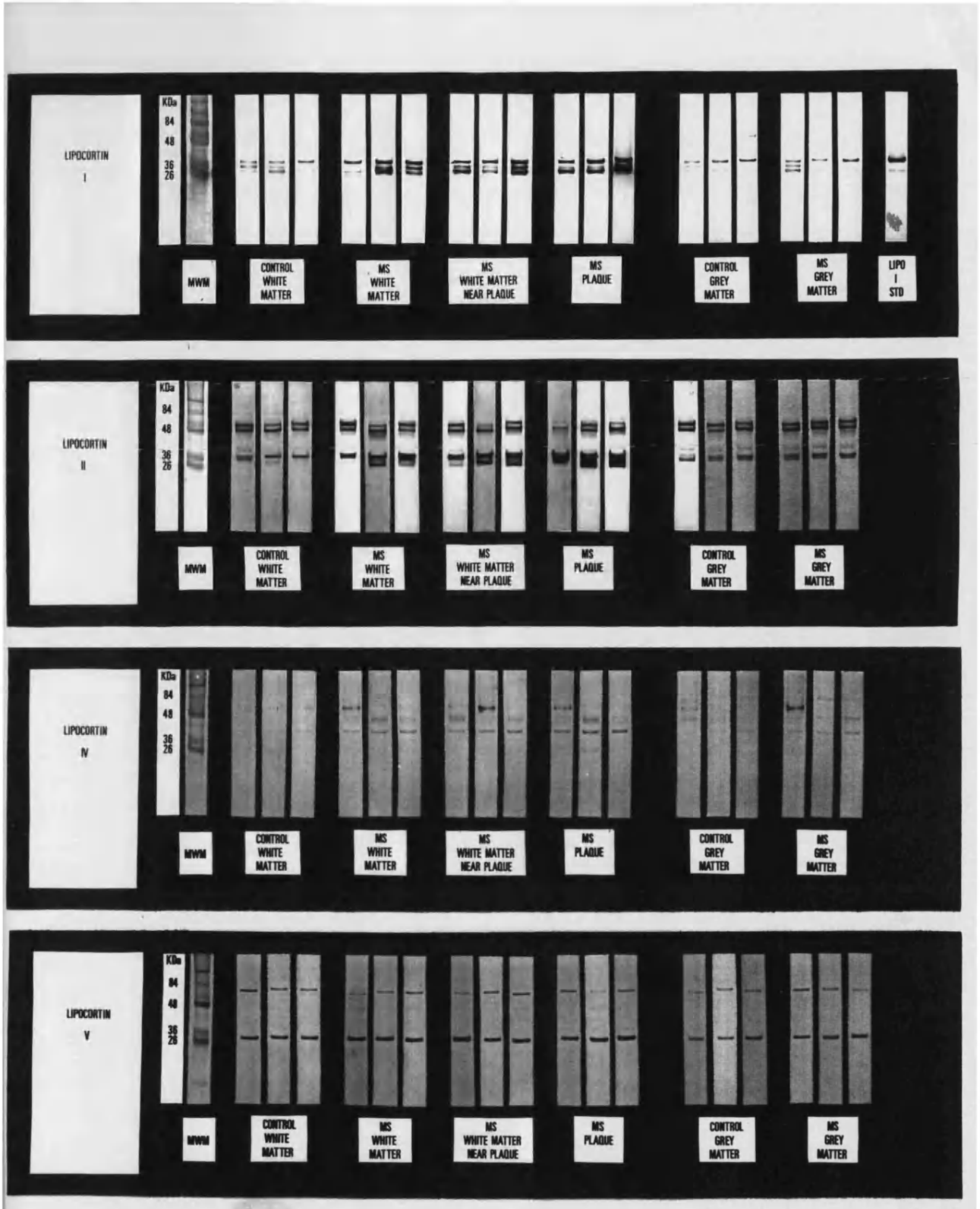


Figure 18. Comparison of lipocortins in normal CNS tissues with lesioned and apparently normal CNS tissues from patients with MS

Western blots of grey and white matter from normal controls and plaque tissue, apparently unaffected white matter adjacent to a plaque, white matter remote from a plaque and grey matter from MS patients were probed for lipocortins I, II, IV and V. In total 35 CNS samples were assayed with 5-7 samples per group, for clarity three representative samples from each group are shown. rh lipocortin I standard was applied at 25 ng per lane. MWM, high molecular mass markers.

Table VII

Densitometer values for western blots of MS and control CNS tissues probed for lipocortins I, II, IV and V.

Tissue		n	Lipocortin I	II	IV	V
White matter –	Control	7	865±247	981±635	109±57	915±272
	MS – remote from plaque	6	2551±1387	1822±1111	468±399	1243±184
	MS – adjacent plaque	5	2585±1111	2771±740[c]	486±168[c]	1356±428
	MS – plaque	6	2842±1308	3086±438	689±548	1530±703
Grey matter –	Control	5	830±276	435±318	78±23	662±318
	MS	6	841±353	612±378	132±44[b]	707±188

*** (I: MS – remote from plaque, MS – adjacent plaque, MS – plaque vs Control)

*** (II: MS – remote from plaque, MS – adjacent plaque vs MS – plaque)

a (II: MS – remote from plaque vs MS – adjacent plaque)

** (IV: MS – remote from plaque, MS – adjacent plaque vs MS – plaque)

* (V: MS – remote from plaque, MS – adjacent plaque, MS – plaque vs Control)

The density of the major band on each immunoblot was assessed using a Joyce-Loebl Chromoscan 3. Results are expressed as mean integral ± standard deviation. For statistical analysis the data were log transformed to stabilise the variance and analysed by One Way Analysis of Variance. Three orthogonal contrasts were fitted for comparison of white matter sub-types.

*P<0.05; **P<0.01; ***P<0.001 for all MS white matter sub-types versus control.

[a]P<0.02 MS white matter remote from plaque versus plaque and adjacent white matter.

[b]P<0.05 MS grey matter versus control grey matter.

n = number of samples per group (except [c], where n = 4).

As can be seen in Figure 18 and Table VII, immunoreactive lipocortins I, II, IV and V were detected in both grey and the white matter samples from normal control subjects and in all other sample types studied. CNS tissues from MS patients appeared to contain markedly increased amounts of all four lipocortins. An overall comparison of all MS white matter subtypes with control white matter showed that immunoreactive lipocortins I, II, IV and V were significantly increased in MS tissue ($P<0.001$, $P<0.001$, $P<0.01$, $P<0.05$ respectively). Moreover, as well as the elevated amount observed in apparently normal MS white matter, levels of each lipocortin were further raised in plaque tissue and in adjacent white matter. Thus within the CNS of individuals with MS there appeared to be a graduation in the tissue content of each lipocortin, with plaque > white matter adjacent to a plaque > white matter remote from a plaque. This pattern was observed for all four lipocortins but reached statistical significance only for lipocortin II. Comparison of control and MS grey matter showed a slight increase in all lipocortins compared to controls, but was only significant at $P<0.05$ for lipocortin IV.

3.2.2 ELISA for Lipocortin I

Since densitometry is only a semi-quantitative technique, an attempt was made to measure the amount of lipocortin I in human CNS samples more accurately using the lipocortin I ELISA. In a preliminary experiment to determine the correct dilution at which to assay the samples, fourteen samples were assessed at four different dilutions corresponding to total protein concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml. Values obtained for the lowest dilution (0.4 mg/ml) fitted best on the linear portion of the standard curve but at all dilutions the majority of samples were within the detection range of the assay. The results for each dilution were normalised by conversion to ng lipocortin I / mg protein and are shown in

Table VIII

Lipocortin I ELISA of CNS tissue: comparison of different sample dilutions

Sample no.	CNS sample type	Total protein concentration of sample at assay mg/ml 0.4	0.2	0.1	0.05
		Lipocortin I ng/mg protein			
1	Control white matter	48	75	125	114
2	MS white matter - adjacent plaque	326	601	731	774
3	MS white matter - plaque	716	1082	1661	2000
4	MS white matter - plaque	310	452	496	530
5	MS white matter - remote from plaque	32	49	37	*
6	Control white matter	*	*	*	*
7	Control grey matter	12	*	*	*
8	MS white matter - remote from plaque	213	252	419	468
9	MS white matter - plaque	407	652	968	838
10	MS white matter - adjacent plaque	324	537	682	826
11	MS grey matter	25	19	*	*
12	MS white matter - adjacent plaque	62	93	143	178
13	MS white matter - plaque	170	238	303	390
14	Control white matter	13	*	*	*

CNS tissue samples from MS patients and controls were homogenised in buffer containing 5 mM EDTA and the supernatants assayed for lipocortin I by ELISA. Each sample was assayed at four different dilutions and the total protein concentration at each dilution is shown at the top of each column. Results are expressed as ng lipocortin I / mg of protein. Values marked with an asterisk were below the detection limit of the assay, 0.5 ng/ml.

Table VIII. As can be seen by comparison of the results in Table VIII with those in Figure 18 and Table VII, at each dilution the overall pattern in the lipocortin I content of the different sample types was very similar to that observed using Western blotting, with the highest amounts found in plaque tissue and the lowest levels in control grey and white matter. However, for any given sample different values were obtained at each dilution: in each case the more concentrated the sample at assay, the lower the apparent lipocortin content.

To investigate whether the presence of CNS tissue homogenate was preventing accurate measurement of lipocortin, four representative samples were assayed at 0.4 mg/ml protein, spiked with either a known amount of rh lipocortin I standard (10 ng) or an equal volume of assay buffer. Table IX shows that values obtained for spiked samples were much lower than expected, with only a fraction of the added lipocortin being detected, suggesting that a component of the sample or homogenisation buffer was interfering with the assay.

To determine the source of this interference a similar spiking experiment was performed to assess the influence of CNS tissue homogenate or homogenisation medium alone on the measurement of lipocortin I. The effect of the amount of lipocortin I originally present in the sample was also investigated. In addition, the influence of media/sample concentration was studied, with both homogenisation medium and CNS samples being assayed at several different dilutions in the presence or absence of 10 ng lipocortin I spike. To account for the possibility that the position of the sample on the plate might influence the results, samples to be tested were sandwiched between two columns of controls comprising 10 ng lipocortin I standard diluted in assay buffer only. Wells were filled one column at a time from left to right. A plan of the plate layout used in this experiment is shown in Figure 19.

Table IX

Lipocortin I ELISA of CNS tissue: effect of samples on measurement of lipocortin

Sample No.	CNS Sample Type	Lipocortin I ng/well		Difference
		-	+	(+)-(-)
1	Control white matter	3.8	4.3	0.5
4	MS white matter-plaque	25.9	17.8	-8.1
7	Control grey matter	0.8	3.7	2.9
11	MS grey matter	1.2	3.8	2.6

CNS tissue supernatants were diluted to 0.4 mg/ml total protein and spiked with either 10 ng rh lipocortin I (+) or an equal volume (10 µl) of assay buffer (-). Unspiked samples were assayed in quadruplicate, spiked samples in duplicate. Results are mean values expressed in ng lipocortin I/well.

	1	2	3	4	5	6	7	8	9	10	11	12
					HM	4	11	HM	4	11		
A	B	4	16	0.06	40 -	40 -	40 -	40 -	40 -	40 -	C	
B	B	2	8	0.03	40 +	40 +	40 +	40 +	40 +	40 +	C	
C	B	1	4	C	20 -	20 -	20 -	20 -	20 -	20 -	C	
D	B	0.5	2	C	20 +	20 +	20 +	20 +	20 +	20 +	C	
E	B	0.25	1	C	10 -	10 -	10 -	10 -	10 -	10 -	C	
F	B	0.13	0.5	C	10 +	10 +	10 +	10 +	10 +	10 +	C	
G	B	0.06	0.25	C	5 -	5 -	5 -	5 -	5 -	5 -	C	
H	B	0.03	0.13	C	5 +	5 +	5 +	5 +	5 +	5 +	C	

Figure 19. Lipocortin I ELISA of CNS tissue: plate plan

Plan of the microtitre plate used to determine the influence of homogenisation medium, CNS tissue homogenate and the lipocortin content of CNS homogenates on the measurement of a known amount of lipocortin I.

Column 1 Blanks (assay buffer only)

Column 2-4B Standard curve: figures show lipocortin/well in µg (column 2) and ng (columns 3 & 4)

Column 4C-H Controls: 10 ng rh lipocortin I per well

Column 5-10 Samples and homogenisation medium: figures represent volume (µl) of sample or medium added to well which for CNS samples corresponded to the following protein concentrations:

40 µl = 0.4 mg/ml
20 µl = 0.2 mg/ml
10 µl = 0.1 mg/ml
5 µl = 0.05 mg/ml

Columns 5 + 8 homogenisation medium

Columns 6 + 9 CNS sample 4, MS plaque rich in lipocortin I

Columns 7 + 10 CNS sample 11, MS grey matter containing little lipocortin I

+, indicated presence of 10 ng/well lipocortin I spike

-, indicates absence

Each well was made up to 100 µl with assay buffer

Column 11 - Controls: 10 ng/well rh lipocortin I standard/well.

Table X

Lipocortin I ELISA of CNS tissue: effect of homogenisation medium, CNS tissue homogenate and the lipocortin content of CNS samples on the measurement of lipocortin I

Sample (plate position)	Protein concentration/ volume	Spike (10 ng)	Lipocortin I ng/well	Apparent value of spike (ng) (+)-(-)
Lipocortin I standard n=6 (column 4)	-	+	8.0	8.0
Homogenisation medium (columns 5 & 8)	40	-	0	
	40	+	14.2	14.2
	20	-	0	
	20	+	10.1	10.1
	10	-	0	
	10	+	7.9	7.9
	5	-	0	
	5	+	6.6	6.6
Sample 4 MS plaque-lipocortin rich (columns 6 & 9)	0.4	-	13.4	
	0.4	+	15.3	1.9
	0.2	-	8.9	
	0.2	+	11.1	2.2
	0.1	-	5.1	
	0.1	+	8.0	2.9
	0.05	-	2.7	
	0.05	+	5.6	2.9
Sample 11 MS grey matter-lipocortin poor (columns 7 & 9)	0.4	-	0.9	
	0.4	+	2.8	1.9
	0.2	-	0.6	
	0.2	+	2.9	2.3
	0.1	-	0.4	
	0.1	+	3.1	2.7
	0.05	-	0.4	
	0.05	+	2.6	2.2
Lipocortin I standard n=8 (column 11)	-	+	5.2	5.2

Legend: Table X

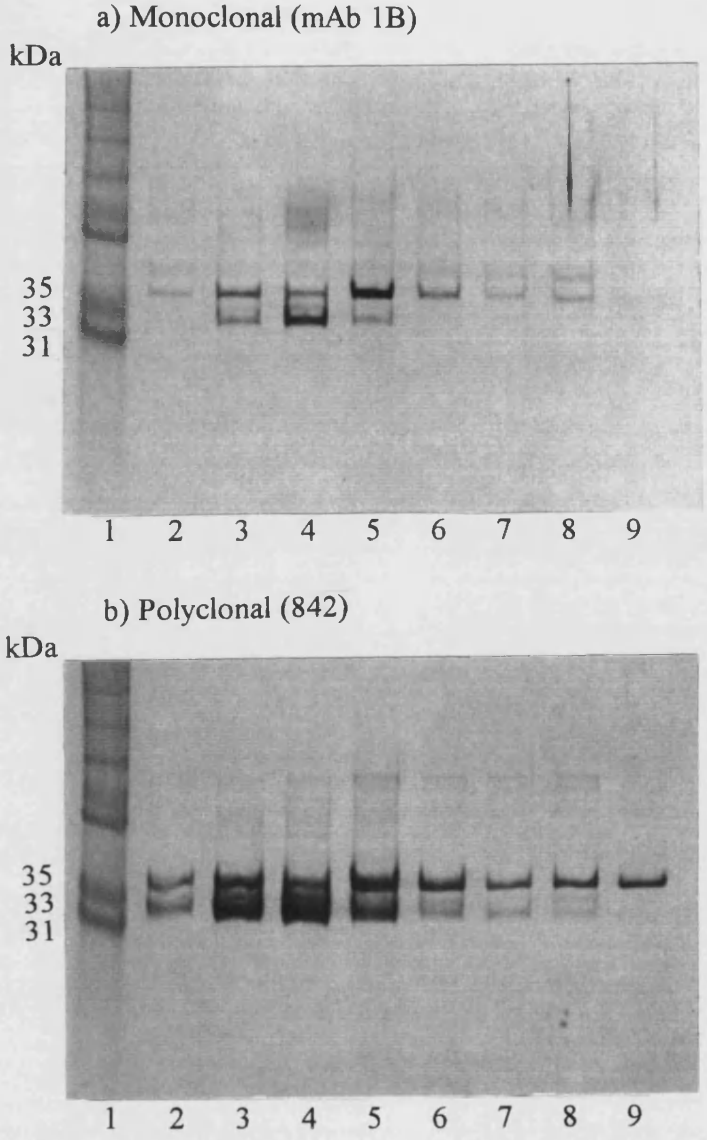
Homogenisation medium and two CNS tissue samples, one rich in lipocortin I and the other containing very little of the protein, were assayed at various dilutions spiked with either 10 ng rh lipocortin I (+), or an equal volume of assay buffer (-). Results are mean values of duplicates (except lipocortin I standard) expressed in ng lipocortin I/well. The second column shows the protein concentration of the sample in mg/ml or the volume of homogenisation medium in µl. The right hand column indicates the apparent value of the 10 ng lipocortin I spike in the presence of medium or CNS samples, which was calculated by subtracting the value obtained for the non-spiked sample from that of the spiked sample.

The results of this experiment are shown in Table X. Values obtained for lipocortin controls were 8.0 ± 1.0 ng (mean ± SD) for column 4 and 5.2 ± 0.5 ng for column 11. Thus even when lipocortin I was diluted in assay buffer only (PBS-Tween) and measured in the absence of media or samples, the observed values were lower than expected, particularly on the right hand side of the plate. However, since the samples were sandwiched between these two columns of controls, the calculated values obtained for the spike would be expected to lie between 8.0 and 5.2 ng if no interference occurred.

Homogenisation medium alone did not appear to inhibit measurement of lipocortin I although the results were quite variable. In the presence of CNS tissue homogenate however, a marked reduction in the detection of lipocortin I was observed, even at relatively dilute protein concentrations. There appeared to be no difference between the effect of a lipocortin rich sample and one containing very little of the protein, thus the amount of lipocortin already present in the sample did not appear to influence recovery.

These results suggested that a factor present in the CNS tissue was interfering with the assay. To determine whether this substance might be a protein, two identical Western blots containing seven typical CNS samples were prepared and each was probed with one of the antibodies used in the ELISA. Figure 20 shows that although there was some very faint staining of higher molecular mass bands, both antibodies (mAb1 and 842) appeared to bind only to lipocortin I and its putative breakdown products to any extent, suggesting that interference was not due to non-specific binding of the antibodies to other proteins in the samples.

CNS tissue has a very high lipid content which could potentially interfere with this assay, either by binding to lipocortin or preventing binding of lipocortin to the monoclonal antibody. To investigate whether this might be the case and examine possible means of prevention, another spiking experiment was performed



Lane	Sample
1	High molecular mass markers
2	Control white matter
3	MS white matter-adjacent plaque
4	MS white matter-plaque
5	MS white matter-plaque
6	MS white matter-remote from plaque
7	Control white matter
8	Control grey matter
9	rh lipocortin I (10 ng)

Figure 20. Specificity of antibodies used in the lipocortin I ELISA
Two identical blots containing representative CNS tissue samples were probed with the antibodies used in the lipocortin I ELISA. (a) monoclonal antibody to lipocortin I (mAb 1B) was used at a dilution of 1:500. (b) polyclonal lipocortin I antisera (842) was diluted 1:5000. Both blots were developed using the peroxidase conjugate technique.

Table XI

Lipocortin I ELISA of CNS tissue: effect of pre-treatment of samples with 5 mM EGTA and/or high speed centrifugation on the measurement of lipocortin I

Sample/Treatment	Spike (20 ng)	Lipocortin I ng/well	Apparent value of spike (+)-(-)
Lipocortin I standard n=5 (column 4)	+	34	34
CNS tissue-Normal preparation	-	0	
	+	11	11
CNS tissue-5 mM EGTA	-	0	
	+	2	2
CNS tissue-100,000 g	-	0	
	+	11	11
CNS tissue-5 mM EGTA & 100,000g	-	0	
	+	0	0
Lipocortin I standard n=8 (column 7)	+	45	45

Rat CNS tissue was homogenised in homogenisation medium in the presence or absence of 5 mM EGTA and aliquots were then centrifuged at either 19,000 or 100,000 g. The supernatants were adjusted to 0.4 mg/ml total protein, spiked with either 20 ng rh lipocortin I (+) or an equal volume (10 µl) of assay buffer (-) and then assayed by ELISA for lipocortin I. Lipocortin I standard was run in columns 4 and 7 and samples in columns 5 and 6. Calculated apparent values for the spike are shown in the column on the right. Values obtained for the lipocortin I standard were higher than expected because the standard curve was not very accurate in this assay run, however this data has been included because the results obtained for the test groups were very clear cut.

using rat CNS tissue. Several methods for pre-treating the samples were employed: chloroform extraction of lipids; centrifugation at 100,0000 g for 1 hour to remove liposomes; and inclusion of 5 mM EGTA in the homogenisation medium to chelate calcium and prevent calcium-dependent binding of lipocortin to phospholipids. Each procedure was tested both alone and in combination with the others. Results are shown in Table XI except for the chloroform treatment, these samples could not be assayed because extraction resulted in the removal of all protein from the samples. As previously observed, measurement of lipocortin I was inhibited in the presence of CNS tissue. High speed centrifugation of samples had no effect on this inhibition, inclusion of 5 mM EGTA resulted in a further reduction in the measurement of lipocortin I.

Due to the problems encountered in attempting to use the ELISA technique to measure lipocortin I in human CNS tissue supernatants, these experiments were not continued.

3.2.3 Localisation of Lipocortin I Immunoreactivity

The cellular localisation of lipocortin I immunoreactivity in normal human CNS tissue and in tissues from patients with MS was investigated using rabbit polyclonal antiserum to rh lipocortin I (code no. 842) and an avidin-biotin complex immunoperoxidase kit which resulted in brown staining of immunoreactive lipocortin I. These experiments were performed by Dr J. Newcombe and a manuscript is in preparation for publication.

3.2.3.1 Normal

In normal control white matter lipocortin I immunoreactivity was located primarily in the walls of blood vessels (Figure 21). In addition, very weak staining of the perikarya of some unidentified cells, which appeared to be scattered

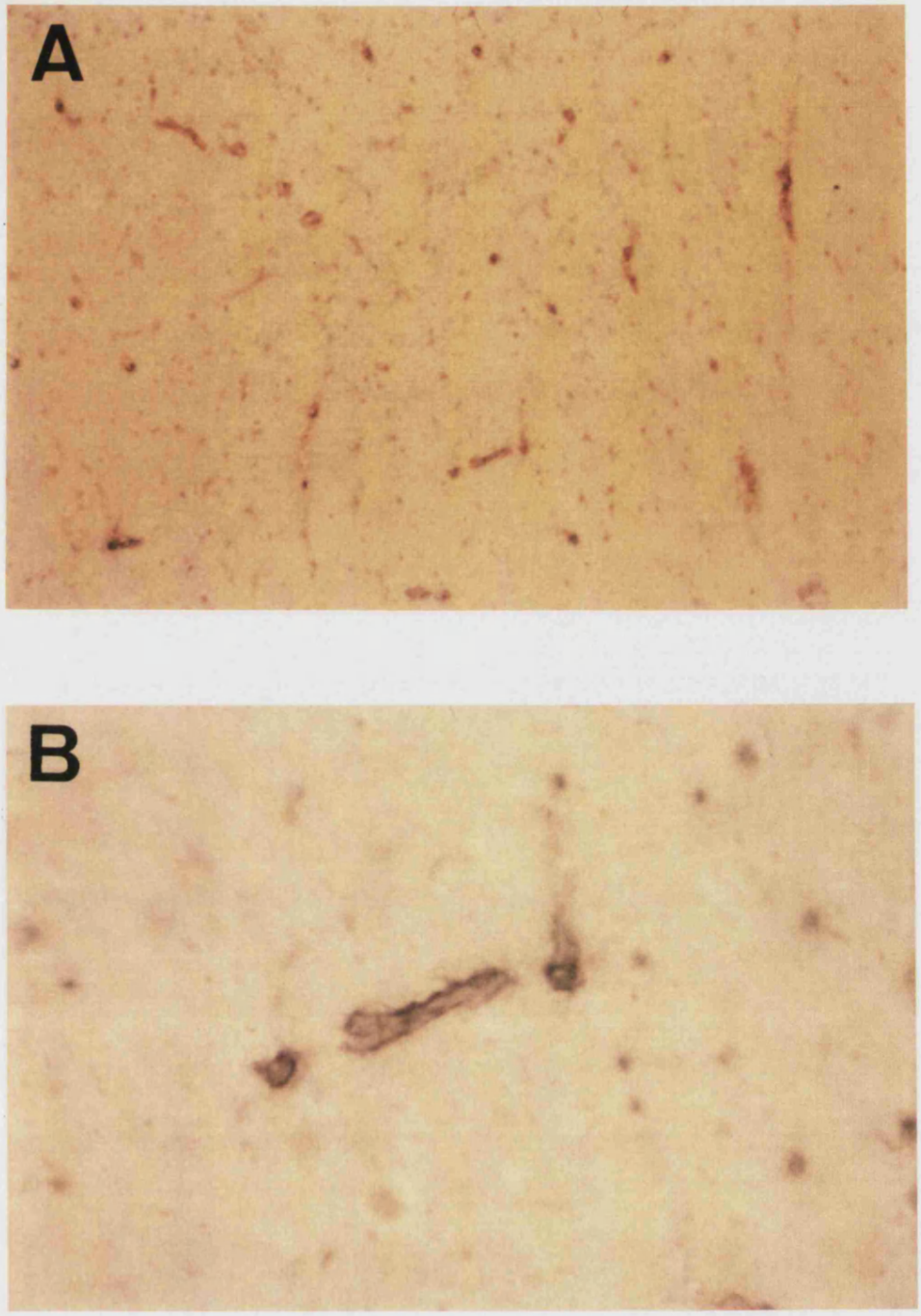


Figure 21. Immunolocalisation of lipocortin I in normal human CNS white matter

Cryostat sections of normal adult human spinal cord white matter, immunoperoxidase stained for lipocortin I. (a) low power field (x187) demonstrating several lipocortin I positive blood vessels, plus very weak staining of the perikarya of unidentified cells. (b) Higher power view of the same section (x750) showing lipocortin I immunostaining of blood vessel walls.

throughout the parenchyma, was also observed. No staining was present on control sections where the primary antibody was omitted or replaced with non-immune rabbit serum.

3.2.3.2 MS

In actively demyelinating MS plaque tissue lipocortin I immunoreactivity was dramatically increased compared to normal control white matter (Figure 22a). This increase was due to widespread staining of small cells which had very strongly stained perikarya, extensive immunostained processes, and were morphologically typical of reactive astrocytes (Figure 22b). In addition, the nuclei, perikarya and processes of small numbers of swollen hypertrophic astrocytes also stained positive for lipocortin I.

For comparative purposes Figure 23 shows adjacent sections from an active plaque immunostained for lipocortin I, the astrocyte marker glial fibrillary acidic protein, and the pan macrophage marker EBM 11. Both anti-lipocortin I and anti-GFAP stained a dense network of cell processes and the similarity in staining patterns strongly suggests that the lipocortin I positive cells in active plaques are astrocytes. In contrast a quite different staining pattern was observed on sections probed with the pan macrophage marker EBM 11. This antibody visualised a large number of reactive macrophages and microglia in actively demyelinating plaques. However on adjacent sections these cells did not appear to be positive for lipocortin I, although immunoreactivity was occasionally observed in monocytes/macrophages in the walls of blood vessels.

In macroscopically normal white matter remote from a plaque, lipocortin I immunoreactivity was slightly increased compared to normal control tissue due to the presence of scattered lipocortin I positive astrocytes. Staining of blood vessels did not appear to change in either plaque tissue or apparently uninvolved white

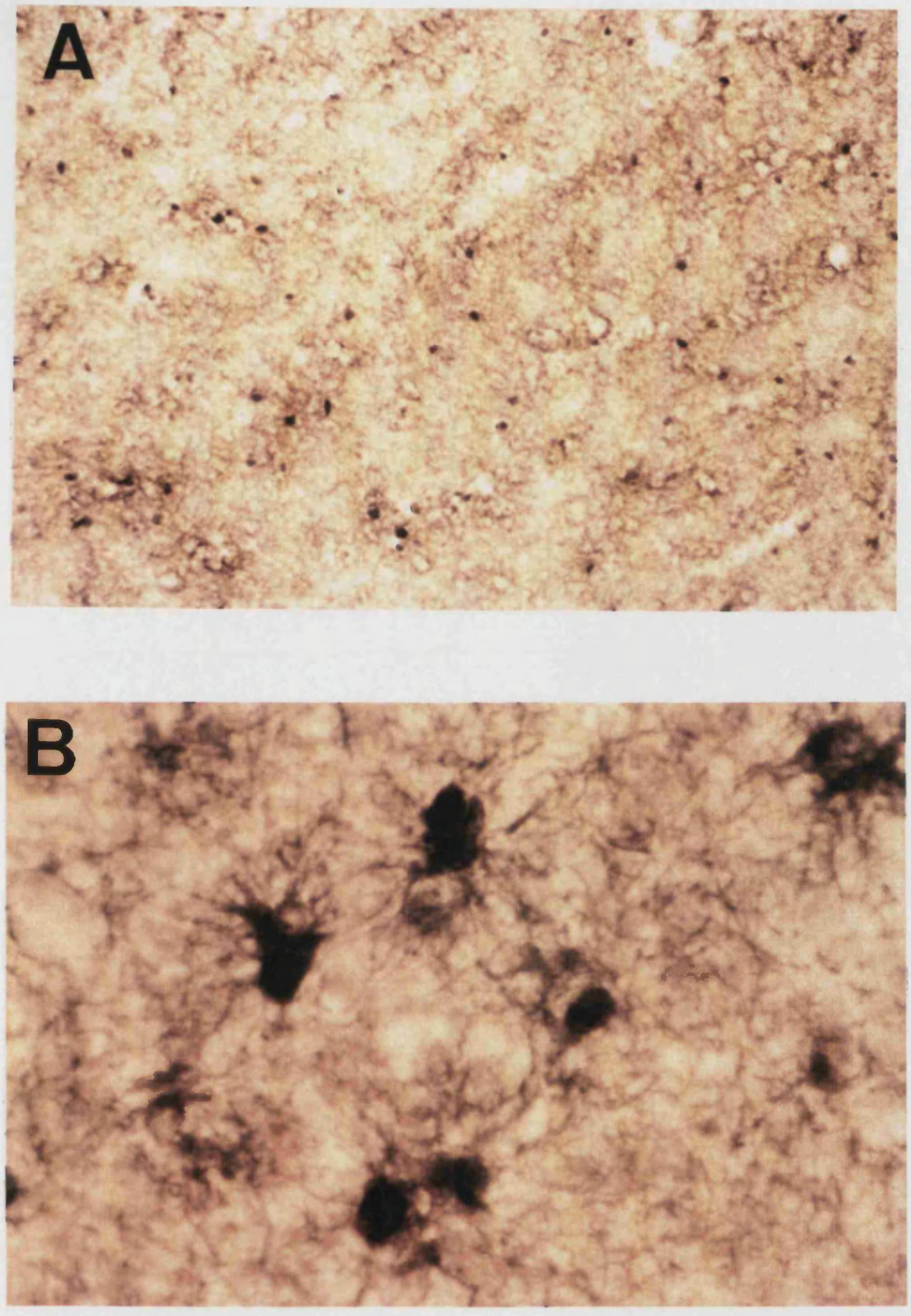


Figure 22. Immunolocalisation of lipocortin I in MS plaque tissue
Cryostat sections of an actively demyelinating plaque in spinal cord white matter, immunoperoxidase stained for lipocortin I. (a) Low power view (x187) showing extensive lipocortin I immunostaining (cf. normal white matter Figure 21a). (b) Higher power view (x750) demonstrating strong staining of astrocyte cell bodies and processes.

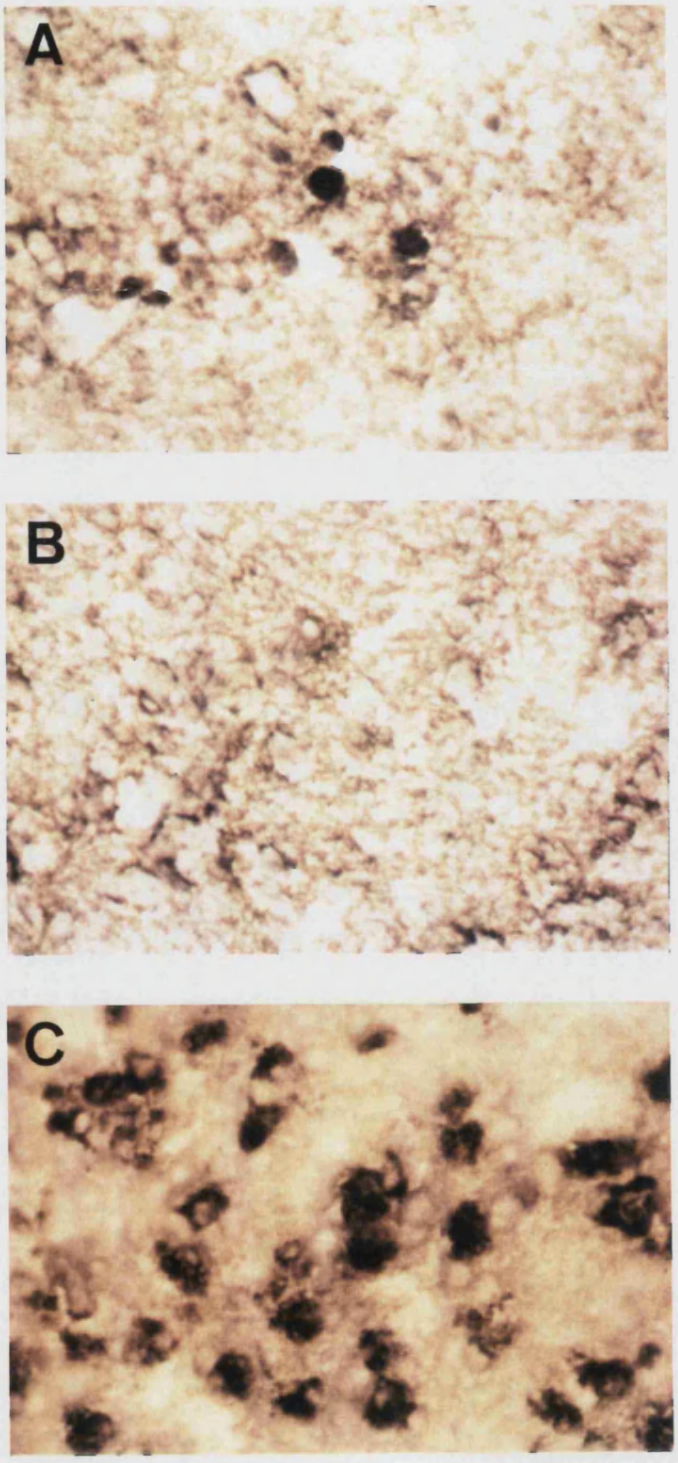


Figure 23. Adjacent sections from an actively demyelinating MS plaque immunostained for lipocortin I, the astrocyte protein GFAP, and the pan macrophage marker EBM 11
(a) Astrocyte perikarya and processes immunostained with lipocortin I antisera. (b) A similar pattern of immunostained cell processes was observed with a monoclonal antibody to the astrocyte marker glial fibrillary acidic protein (GFAP). (c) Activated macrophages and microglia immunostained for the pan macrophage marker EBM 11, these cells did not appear to be positive for lipocortin I. All at magnification x750.

matter. Control sections of MS plaque tissue were negative when the primary antibody was omitted or replaced with non-immune rabbit serum.

PART B: LIPOCORTINS IN THE RAT CNS DURING EAE

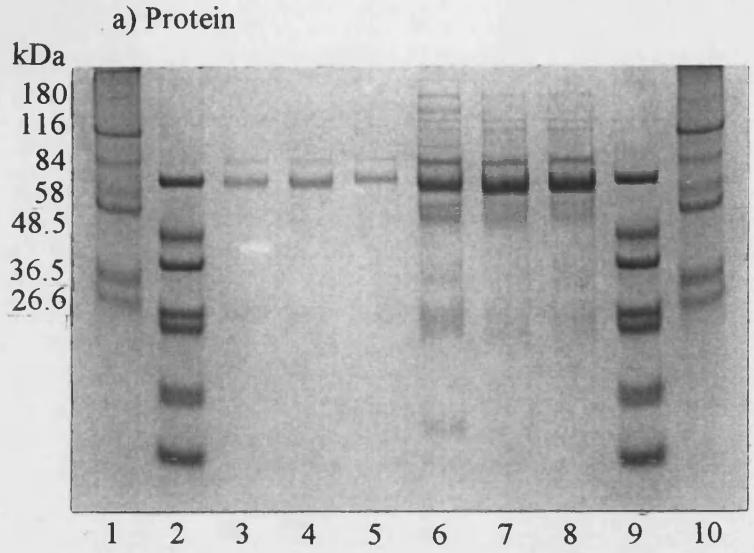
3.3 CEREBROSPINAL FLUID

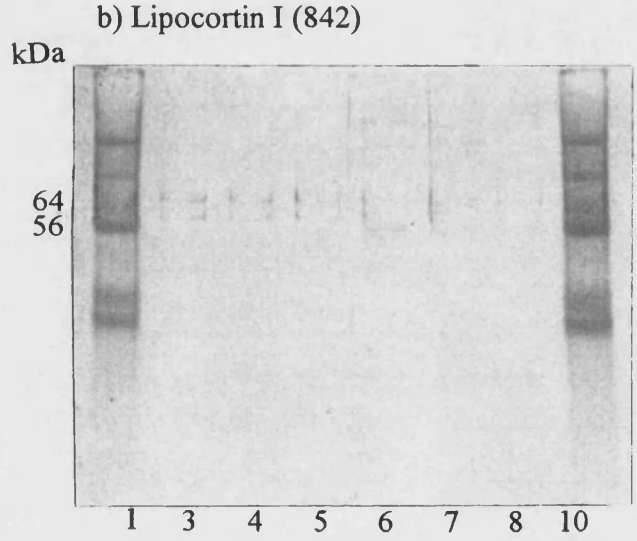
3.3.1 Cell-free Supernatants

Western blotting was used to investigate the lipocortin content of cell-free CSF supernatants taken from normal rats and EAE-inoculated animals at the height of disease and immediately following recovery. Samples were run individually and a gel stained for total protein showed that all the samples contained minimal amounts of protein, although slightly increased levels were observed in CSF from EAE-inoculated animals (Figure 24). However, when samples were immunoblotted for lipocortin I using the polyclonal antiserum code no. 842 and the peroxidase conjugate technique, lipocortin I was undetectable. Very faint bands at 64 and 56 kDa were present in each sample but appeared to be similar in all animals regardless of treatment.

3.3.2 Whole CSF

The same technique was also used to investigate lipocortin I in lysate of whole CSF. Samples were collected from normal and CFA-inoculated controls and EAE-inoculated animals during the induction, diseased and recovery phases. In a preliminary experiment samples were run individually to assess variation within groups, and results of this experiment are shown in Figure 25. Lipocortin I was not detectable in the CSF of normal and CFA-inoculated controls, nor in CSF





Lane	Sample
1	High molecular mass markers
2	Low molecular mass markers
3	Normal
4	Normal
5	Normal
6	EAE-diseased
7	EAE-diseased
8	EAE-recovered
9	Low molecular mass markers
10	High molecular mass markers

Figure 24. Immunoblot for lipocortin I in cell-free CSF from normal and EAE-inoculated Lewis rats

(a) Protein-stained gel and (b) Western blot of cell-free CSF supernatants from normal, EAE-diseased and EAE-recovered rats, probed for lipocortin I using antibody code number 842.

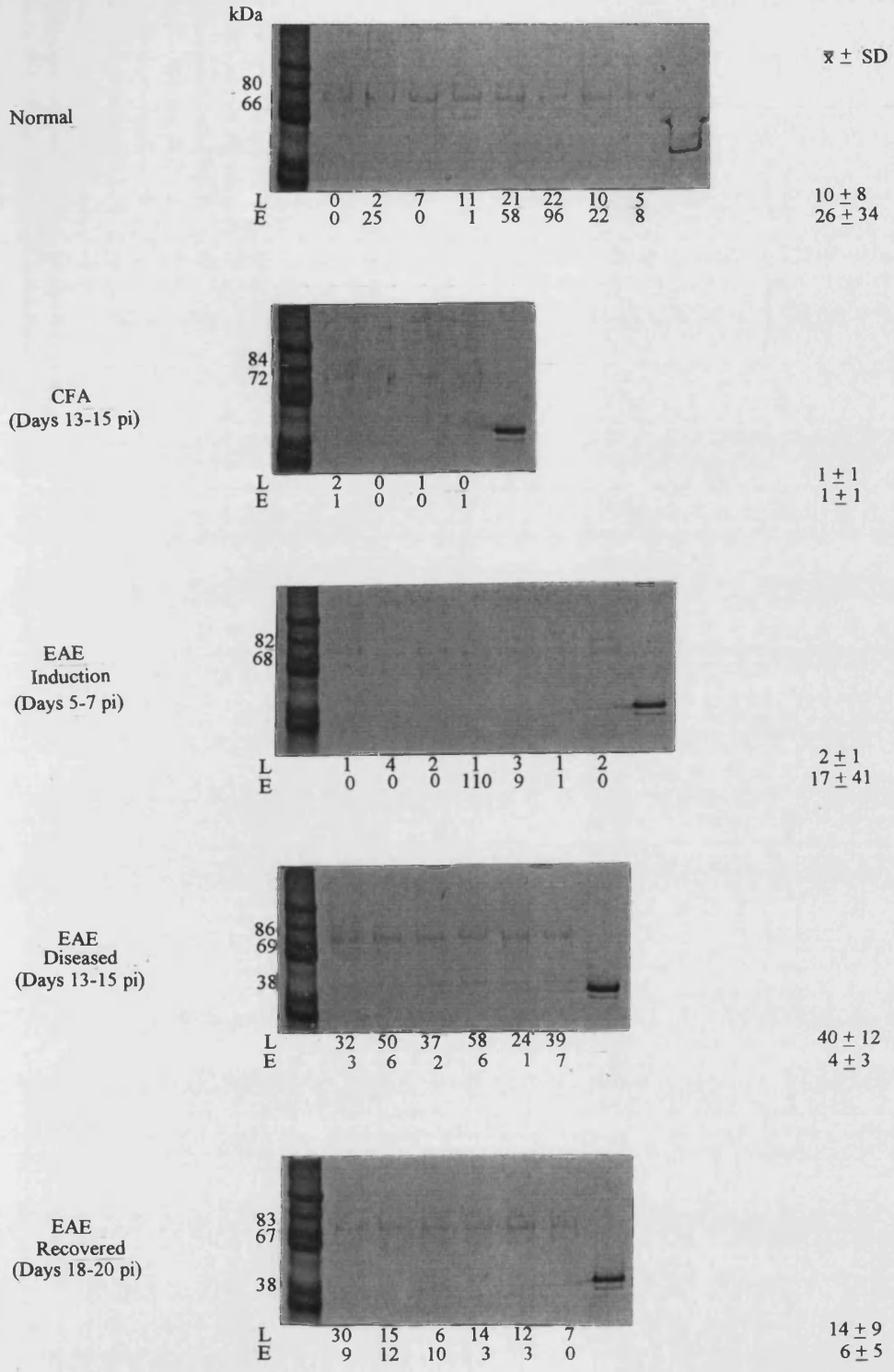


Figure 25. Lipocortin I in the CSF of individual Lewis rats during various stages of EAE
Western blots of whole CSF lysate taken from normal, CFA-inoculated and EAE-inoculated animals were probed with lipocortin I antiserum code no. 842. Days post-inoculation (pi) on which the animals were sampled are shown in parenthesis. The first lane of each blot contains high molecular mass markers, subsequent lanes, CSF from individual animals, and the final lane of each blot was loaded with 50 ng rh lipocortin I. The figures below each lane show the CSF leukocyte count (L) and erythrocyte count (E) in cells/ml ($x10^4$). Mean counts for each group are shown on the right.

taken from EAE-inoculated animals during the induction phase. However, small amounts of an approximately 38 kDa lipocortin I-like immunoreactive protein were present in the spinal fluid of four out of six animals with neurological symptoms of EAE and two out of six animals which had recently recovered from the disease. In addition, two very faint bands at approximately 83 and 68 kDa were observed in all samples but these did not appear to change significantly throughout the course of the disease.

The groups in which the lipocortin I-like protein was detected had higher CSF leukocyte counts than groups where the protein was absent, although for individual samples within these groups there appeared to be no relationship between cell number and the presence or absence of the protein. There was no correspondence between the occurrence of lipocortin and erythrocyte number.

Since this initial experiment revealed only minimal variation in the lipocortin content of samples within the same group, pooled spinal fluid samples were used in subsequent experiments. This allowed all groups to be run on the same blot, thus eliminating errors which may arise from comparison of samples processed on different blots.

Two separate experiments were performed to assess lipocortins in pooled samples of CSF lysate. Due to the low levels previously found in CSF, the more sensitive PAP-Ni/Co method of immunoblotting was developed and used for the detection of lipocortins in these samples. The results of one such experiment are shown in Figure 26. In the first experiment lipocortins appeared to be below the limit of detection in spinal fluid from normal, CFA controls and EAE-inoculated animals during the induction phase. However, in the second experiment extremely low levels of a 37 kDa lipocortin I-like protein were detected in these groups. In each case increased levels of the protein were found in CSF taken from EAE-diseased rats and a slightly reduced amount in the CSF of convalescent animals. In

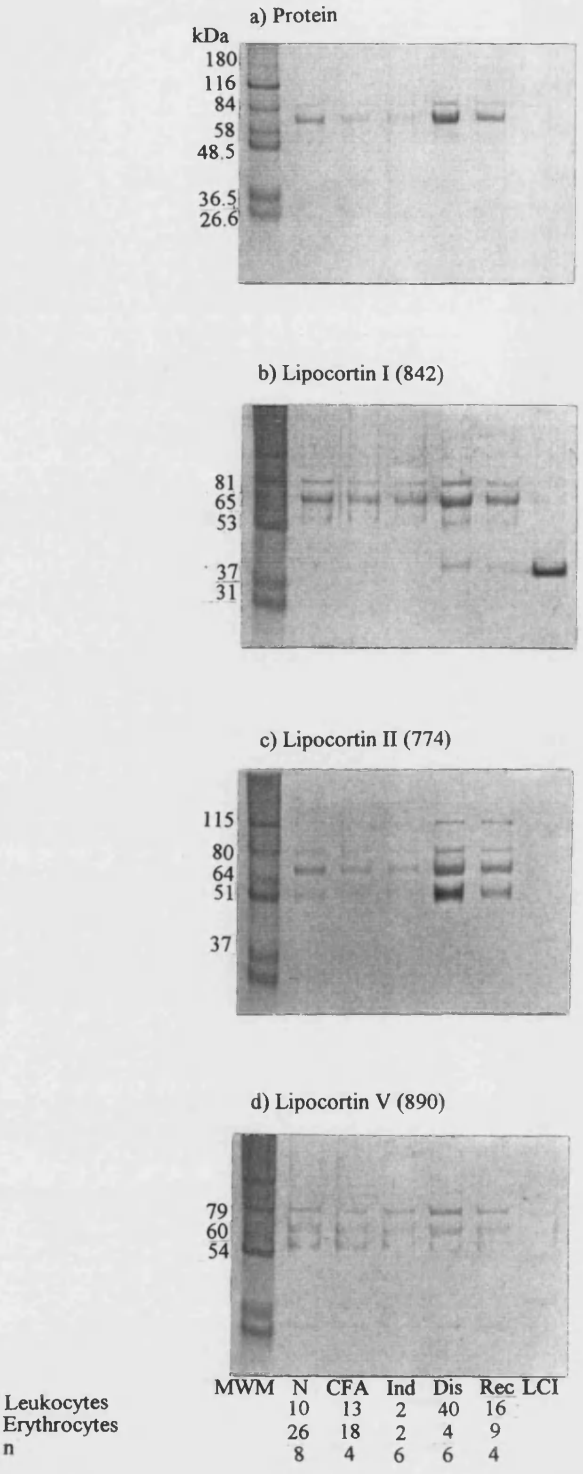


Figure 26. Lipocortins in pooled CSF taken from Lewis rats during various stages of EAE
(a) protein-stained gel and (b-d) Western blots of whole CSF lysate pooled from groups of normal (N), CFA-inoculated (CFA), and EAE-inoculated rats during the induction (Ind), disease (Dis), and recovery (Rec) phases, probed for lipocortins I, II and V. Antibody code numbers are given in parenthesis. Figures below each lane show the mean number of leukocytes and erythrocytes per ml ($x10^4$) and the number of samples per group (n). The final track of blot b contains 10 ng rh lipocortin I standard (LCI).

addition, very low levels of a 31 kDa lipocortin I immunoreactive protein were also detected in these samples. Again the increased amount of immunoreactive lipocortin I in the disease and recovery groups appeared to coincide with an elevation in the CSF leukocyte count and there seemed to be no concordance with CSF erythrocyte number.

In contrast immunoreactive proteins corresponding to the molecular weight of lipocortins II and V were not detected in CSF from either normal or EAE-inoculated animals. Interestingly a band of approximately 51 kDa was observed on the blot probed for lipocortin II which was markedly increased in EAE-diseased animals. Several very faint bands between 50 and 80 kDa were present on all blots, some of which also appeared to increase during EAE.

Although the method used to collect rat CSF was adequate for obtaining samples from control animals, it proved very difficult to consistently obtain sufficient volumes of CSF from rats with EAE. In each experiment 6-8 animals were inoculated for EAE per group, but often far fewer samples were collected. Due to the difficulty in obtaining sufficient volumes of spinal fluid from rats with EAE, and the very low levels of lipocortin found in CSF samples these experiments were not continued.

3.4 CNS TISSUE

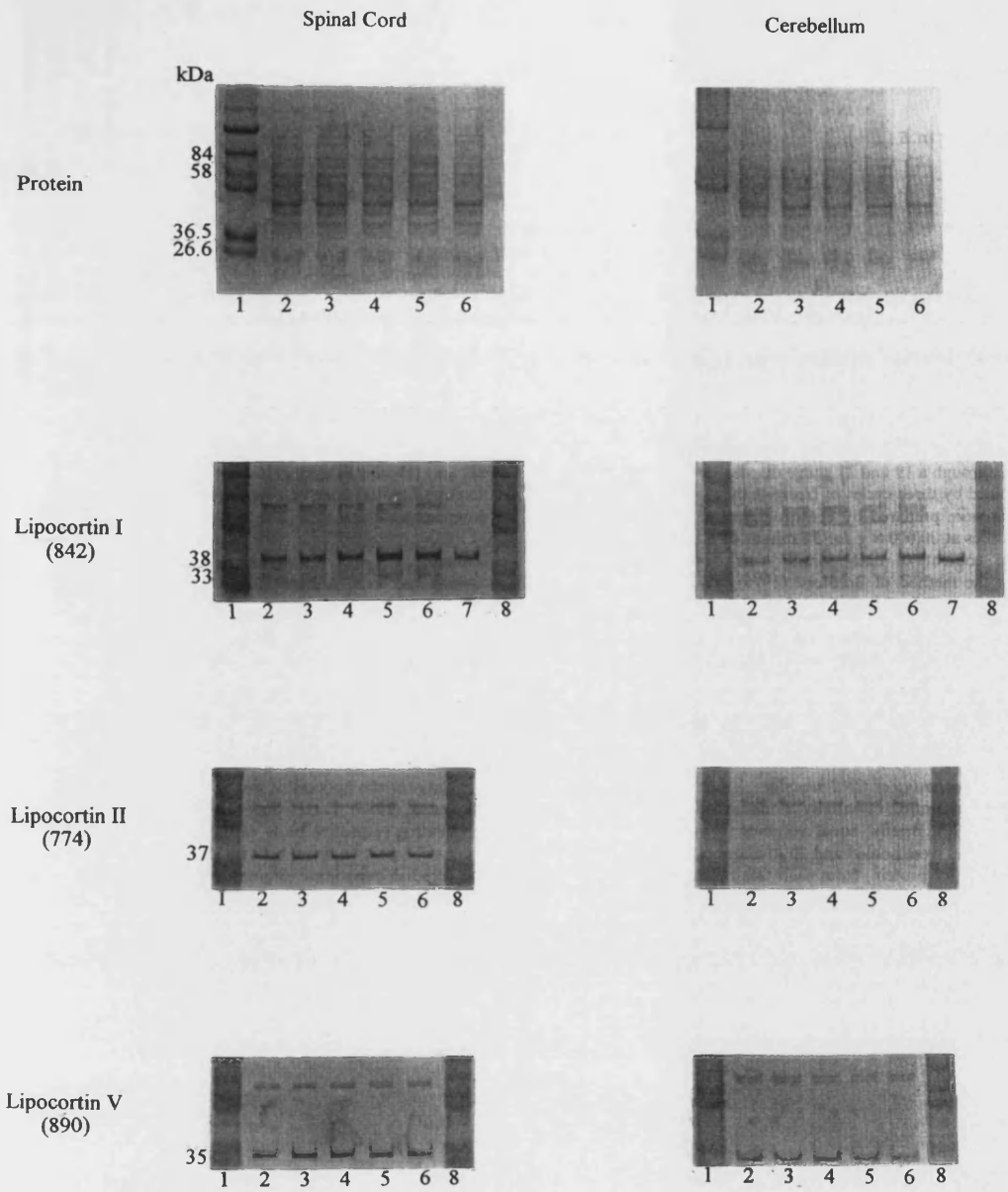
3.4.1 Detection of Lipocortins I, II and V

These experiments were performed in conjunction with Dr C. Bolton and have been published in the Journal of Neuroimmunology (Bolton *et al.* 1990). To investigate the presence of lipocortins in the CNS of Lewis rats during the course of EAE, cervical spinal cords and cerebella were collected from normal, CFA-

inoculated controls and EAE-sensitised rats during the induction, diseased and recovery phases. CNS tissues were bisected down the midline and one half was sectioned and stained with H & E in order to estimate tissue lesion load while the other portion was probed for lipocortins I, II and V using Western blotting and the peroxidase conjugate technique. In a preliminary immunoblotting experiment samples were run individually, since this revealed little variation in lipocortin content within the groups (not shown), samples were subsequently pooled to enable all groups to be run on one blot. Two separate identical experiments were performed with six animals per group and essentially the same results were observed in each. Results for the second experiment are shown in Figure 27.

Immunoreactive bands corresponding to the molecular masses of lipocortins I, II and V were detected in both the cervical spinal cord and cerebellum of normal Lewis rats. On blots probed for lipocortin I the major band was approximately 38 kDa and co-migrated with rh lipocortin I standard. A lower molecular weight band of approximately 33 kDa was also observed which appeared to be proportional to the main band. The principle bands on blots probed for lipocortins II and V were approximately 37 and 35 kDa respectively which is similar to the molecular mass reported for these proteins by Pepinsky *et al.* (1988).

As can be seen in Figure 27, levels of lipocortin I-like protein were slightly increased in the cervical spinal cord of CFA-inoculated animals compared to normal controls, and a similar elevation was observed in cords taken from EAE-inoculated rats during the induction phase. Onset of symptoms was accompanied by a further marked increase in immunoreactive lipocortin I in the spinal cords of physically sick animals, with a slight decrease in convalescent rats. In the cerebellum a similar pattern was observed, with raised levels of the protein present in CFA-inoculated controls and in EAE-inoculated pre-diseased and diseased animals. However changes in the cerebellum were not as great as those in the



Lane	Sample
1	High molecular mass markers
2	Normal controls
3	CFA controls
4	EAE-inoculated: induction
5	EAE-inoculated: diseased
6	EAE-inoculated: recovered
7	rh lipocortin I (25 ng)
8	High molecular mass markers

Figure 27. Lipocortins in the cervical spinal cord and cerebellum of Lewis rats throughout the course of EAE
Protein-stained gel and Western blots of pooled rat CNS tissue supernatants probed for lipocortins I, II and V. Spinal cords and cerebella were taken from normal and CFA-inoculated controls and from EAE-inoculated rats during the induction, diseased and recovery phases (n=6). Antibody code numbers are shown in parenthesis.

Table XII

Perivascular infiltrates in CNS tissues during the course of EAE

Treatment	Mean number of lesions per section (± SD) Spinal Cord	Cerebellum
Normal	ND	ND
CFA	0	0
EAE - Induction	0	0
EAE - Diseased	54±13	2±2
EAE - Recovery	29±10	4±3

Sections were cut at one standard depth, stained with H & E and the total number of lesions per section was determined using light microscopy. Values are means of six animals ± SD. ND, not done.

spinal cord and there appeared to be a further slight increase in lipocortin I immunoreactivity in this region following recovery.

In contrast to the results obtained for lipocortin I, the levels of lipocortins II and V did not appear to change in either CFA or EAE-inoculated animals. On all blots fainter bands between 62 and 70 kDa were observed although the pattern of distribution remained unchanged throughout the course of EAE.

Increases in the lipocortin I content of the cervical spinal cord and cerebellum of EAE-inoculated rats coincided with the appearance of inflammatory lesions in these tissues (Table XII). In both CNS areas, lesions were absent during the induction period. In the cervical spinal cord, large numbers of perivascular infiltrates were present during the disease phase with a reduced amount following recovery, whereas in the cerebellum low numbers of lesions were found in diseased animals, which appeared to be slightly increased in rats which had recently recovered.

3.4.2 Distribution of Lipocortin I

To investigate the cellular localisation of lipocortin I immunoreactivity in the CNS throughout the course of EAE, polyclonal antiserum raised against rh lipocortin I (842) and PAP immunohistochemistry were used. The presence of immunoreactive-lipocortin I was revealed by brown staining and to aid identification of cells, sections were lightly counterstained with toluidine blue. In two separate experiments the distribution of lipocortin I immunoreactivity was studied in cervical spinal cord sections from normal and CFA-inoculated controls, and from animals inoculated for EAE. EAE-sensitised rats were sampled during the pre-clinical phase on days 4 and 7 PI, during the disease stage on the first day of exhibiting either weight loss, flaccid tail, hind limb weakness or paralysis and during the recovery phase on the first day of complete absence of neurological

signs. Serum samples were also collected from these animals for measurement of corticosterone. The results of this work have been published in the Journal of the Neurological Sciences (Elderfield *et al.* 1993).

3.4.2.1 Normal

In cervical spinal cord sections from normal animals, lipocortin I immunoreactivity was localised predominantly in the walls of larger blood vessels (Figure 28a,b). However staining was patchy and many blood vessels appeared unstained or only weakly positive. Some capillaries were also very faintly stained (Figure 28c), although most were at or below the visual limit of detection. Occasionally very heavily stained cells were seen within the lumen of blood vessels and capillaries, three such cells are shown in Figure 28d. The nuclei of many neurones also appeared to be faintly stained (Figure 28a) but this proved to be non-specific (see below). No staining of oligodendrocytes or astrocytes was observed.

Control sections from normal animals are shown in Figure 29. When lipocortin I antiserum was omitted or replaced with non-immune rabbit serum, sections were completely negative (Figure 29b,c). However when sections were incubated with antiserum which had been pre-adsorbed with 100 μg/ml rh lipocortin I, staining of vascular structures and intravascular cells was inhibited, but staining of neuronal nuclei was still present indicating that this was non-specific (Figure 29d,e).

3.4.2.2 EAE

Typical sections taken from EAE-inoculated animals at various stages of disease and immunostained for lipocortin I, are shown under low magnification in Figure 30. In the cervical spinal cord of EAE-inoculated rats, prior to the onset of neurological symptoms, the distribution and intensity of lipocortin I

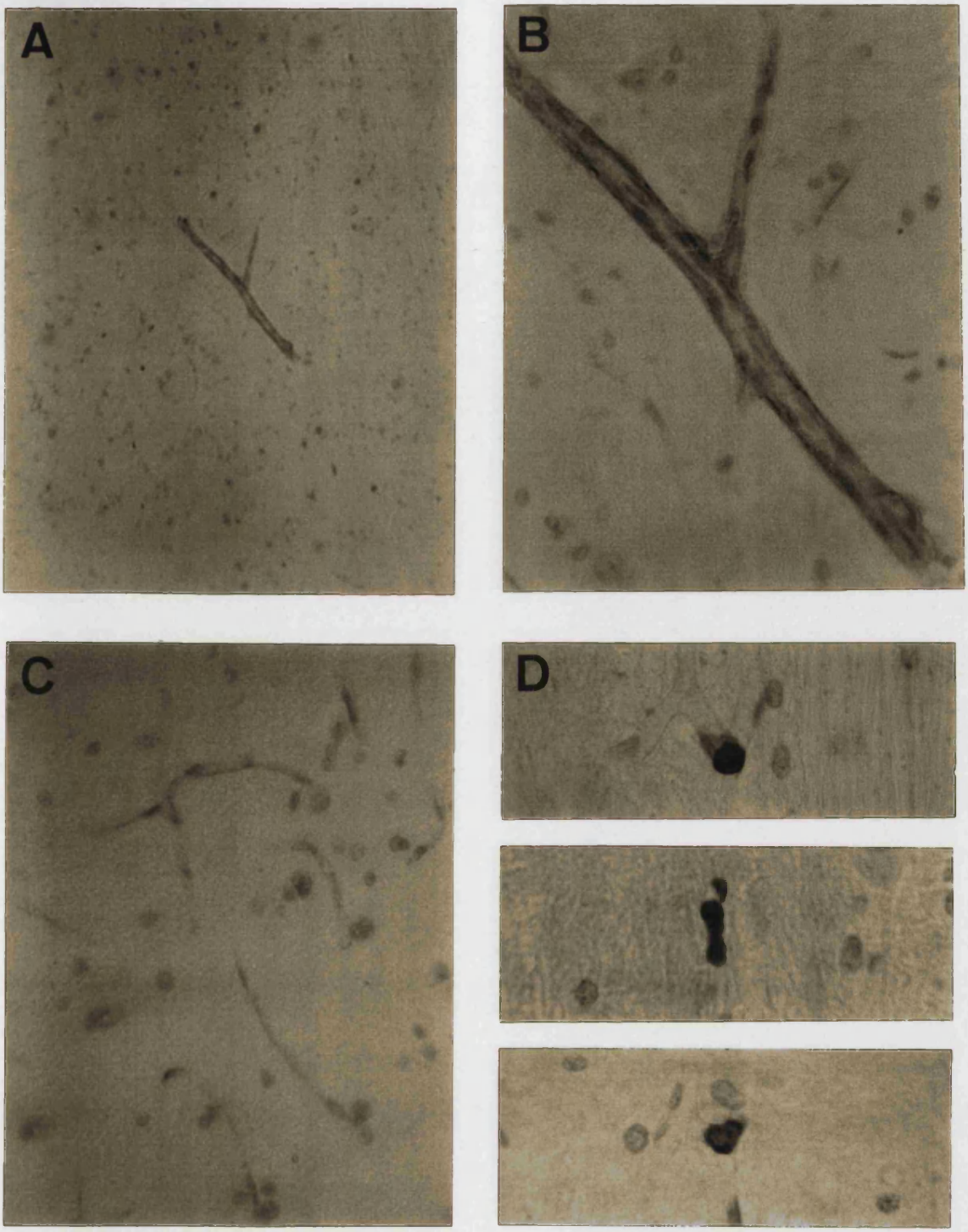


Figure 28. Immunolocalisation of lipocortin I in the cervical spinal cord of normal Lewis rats
(a) Low power view (x100) showing localisation of lipocortin I immunoreactivity in the walls of blood vessels plus very faint non-specific staining of neuronal nuclei. (b,c) Higher power fields (x400) of lipocortin I positive blood vessels and capillaries respectively. (d) Three typical examples of the very heavily stained cells which were occasionally observed within the lumen of blood vessels (x600).

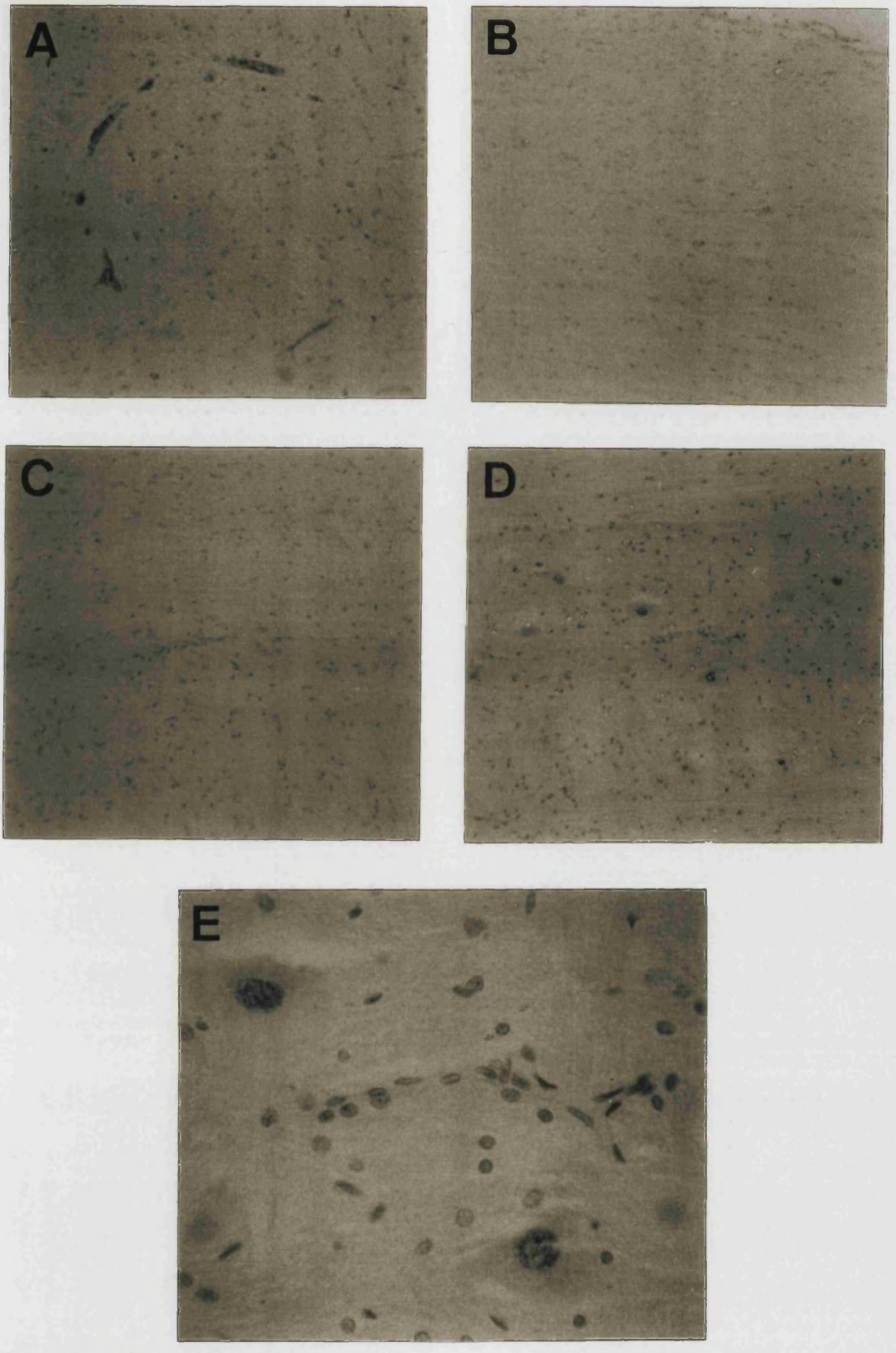


Figure 29. Control sections: normal CNS tissue
Cervical spinal cord sections from normal Lewis rats incubated with: (a) lipocortin I antisera, positive control showing immunostained blood vessels (x100); (b) PBS only, no primary antibody, negative control (x100); (c) non-immune rabbit serum (x100); (d) lipocortin antiserum pre-adsorbed with 100 μg/ml rh lipocortin I. (e) Higher power view of d demonstrating an unstained blood vessel and non-specific staining of neuronal nuclei (x400).

immunoreactivity was the same as that found in normal animals. However the onset of symptoms and the appearance of inflammatory lesions coincided with a marked increase in lipocortin I immunostaining. As the disease progressed the extent of staining increased, and appeared to be proportional to the number of lesions and the severity of symptoms. Cords from rats exhibiting weight loss or flaccid tail had only a few small lipocortin-stained lesions, whereas progression of symptoms to hind limb weakness and paralysis was characterised by widespread staining of numerous inflammatory infiltrates. Generally, fewer lipocortin-stained lesions were present in sections taken from animals which had recently recovered from paralysis. Sections from CFA-inoculated controls showed the same distribution and degree of staining as normal and pre-diseased animals.

Staining of CNS lesions was quite variable: whilst a few were totally unstained, in many more all infiltrating cells appeared to be positive for lipocortin I. In general however, lipocortin I immunoreactivity was present in the vast majority of cells constituting the inflammatory infiltrates. Several typical lipocortin-stained lesions in sections taken from animals displaying complete paralysis are shown in Figure 31 and two representative perivascular infiltrates are shown under higher magnification in Figure 32. Most of the cells were moderately stained and possessed the morphological characteristics of lymphocytes (small and round with a round nucleus surrounded by a thin rim of cytoplasm) and macrophages (larger, irregularly shaped, with an irregular nucleus and a higher cytoplasmic to nuclear ratio). In addition, some very heavily stained cells, which were large and heterogenously shaped, were observed in association with the cellular infiltrates and often some distance into the surrounding parenchyma (Figures 31 & 32). These cells appeared to be similar to some of those occasionally seen within the blood vessels of normal animals (Figure 28d). Their size and morphology suggests that these are also macrophage-type cells, although identification was difficult

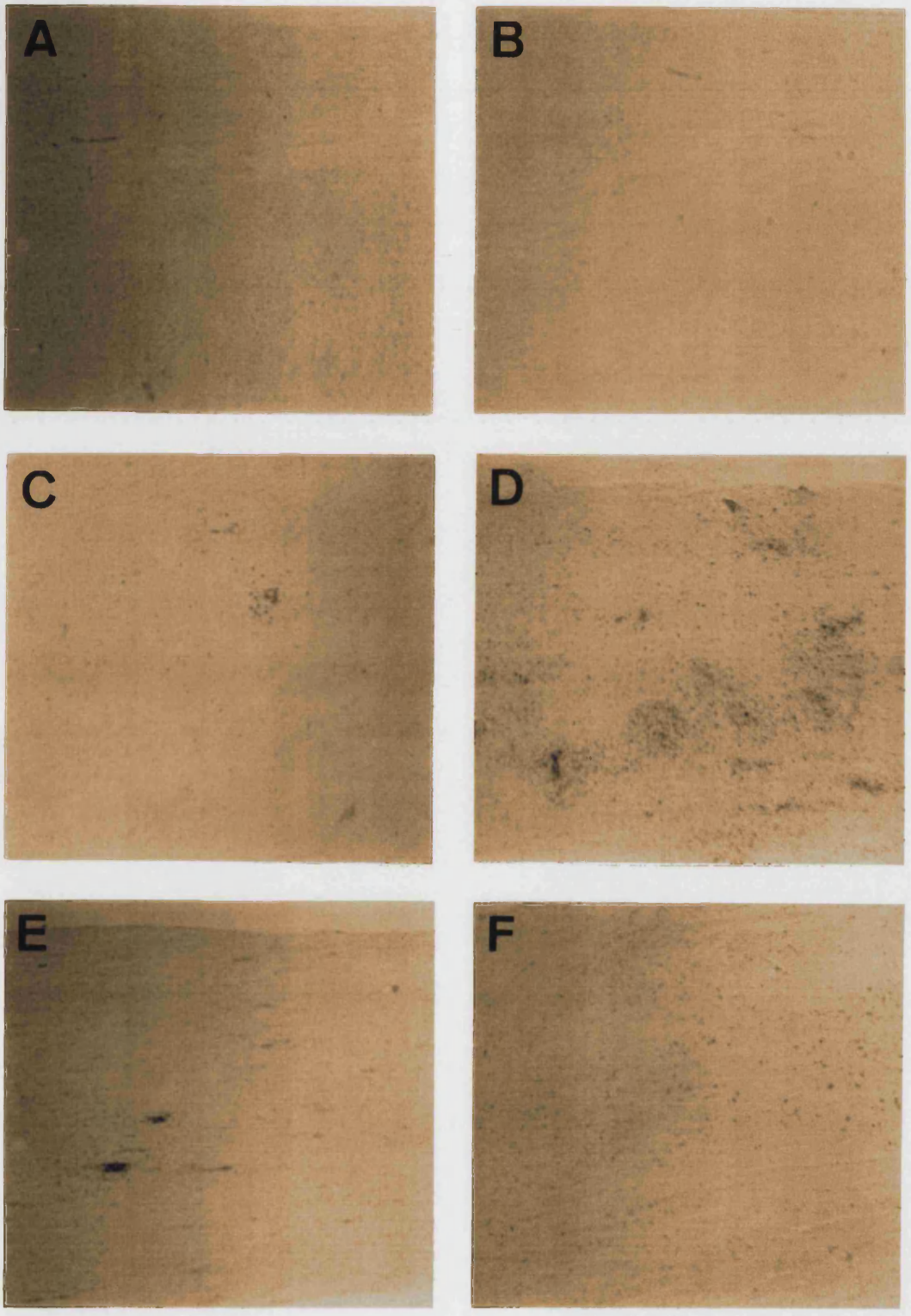


Figure 30. Lipocortin I immunoreactivity in cervical spinal cords taken from Lewis rats at various stages of EAE

Low power views (x40) of representative sections taken from EAE-inoculated animals during the pre-diseased phase on days 4 (a) and 7 (b) post-inoculation, during the diseased stage from animals displaying signs of flaccid tail (c) and complete paralysis (d) and immediately after recovery from EAE (e). (f) section from CFA-inoculated rat taken on day 15 post-inoculation.

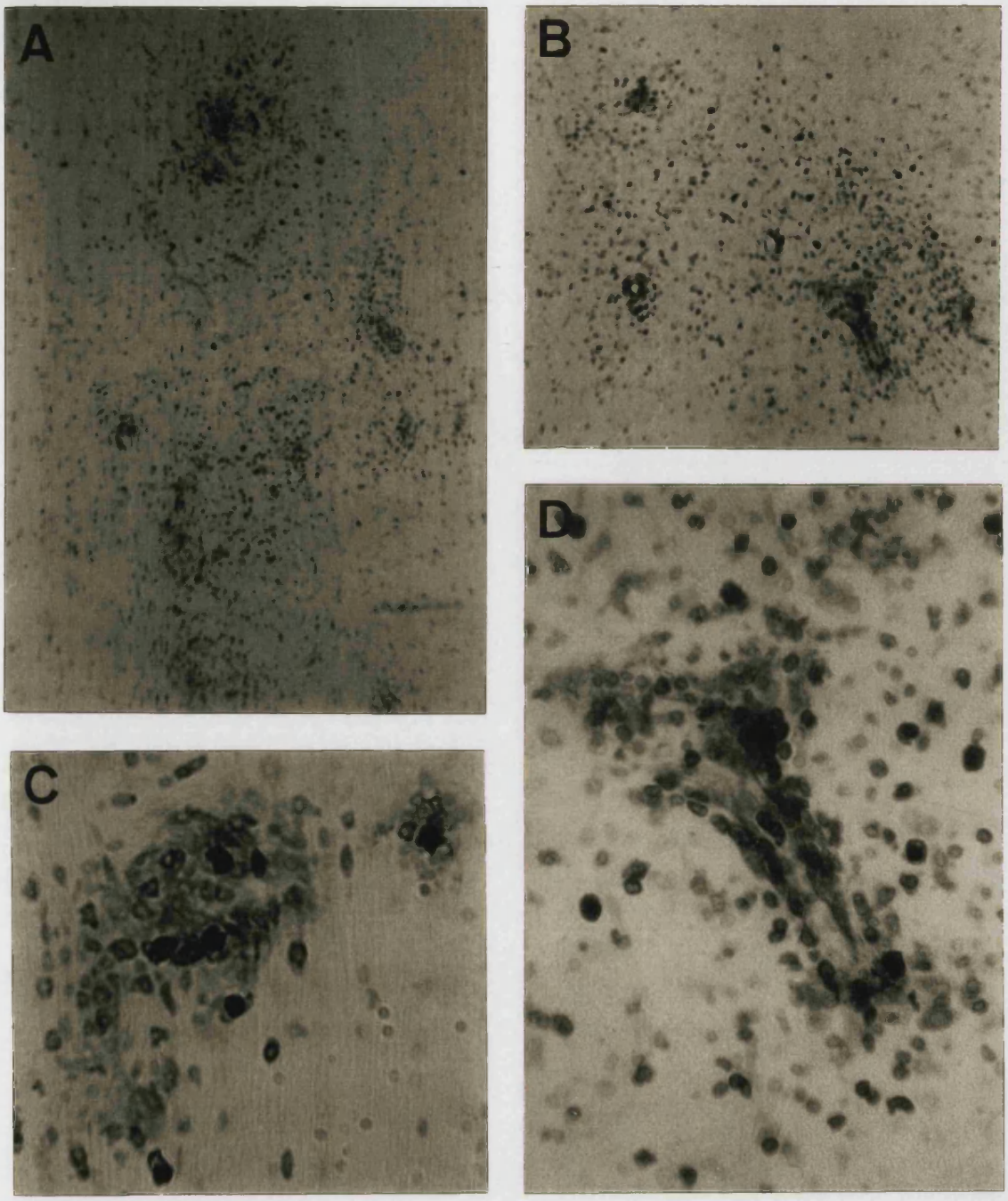


Figure 31. Lipocortin I immunostaining of inflammatory lesions in the cervical spinal cord of EAE-diseased rats

All sections are from rats displaying symptoms of complete paralysis. (a,b) Low power views (x100) showing extensive immunostaining of several inflammatory lesions. (c,d) Higher power fields (x400) of typical perivascular lesions demonstrating that the vast majority of infiltrating cells appear to be positive for lipocortin I.

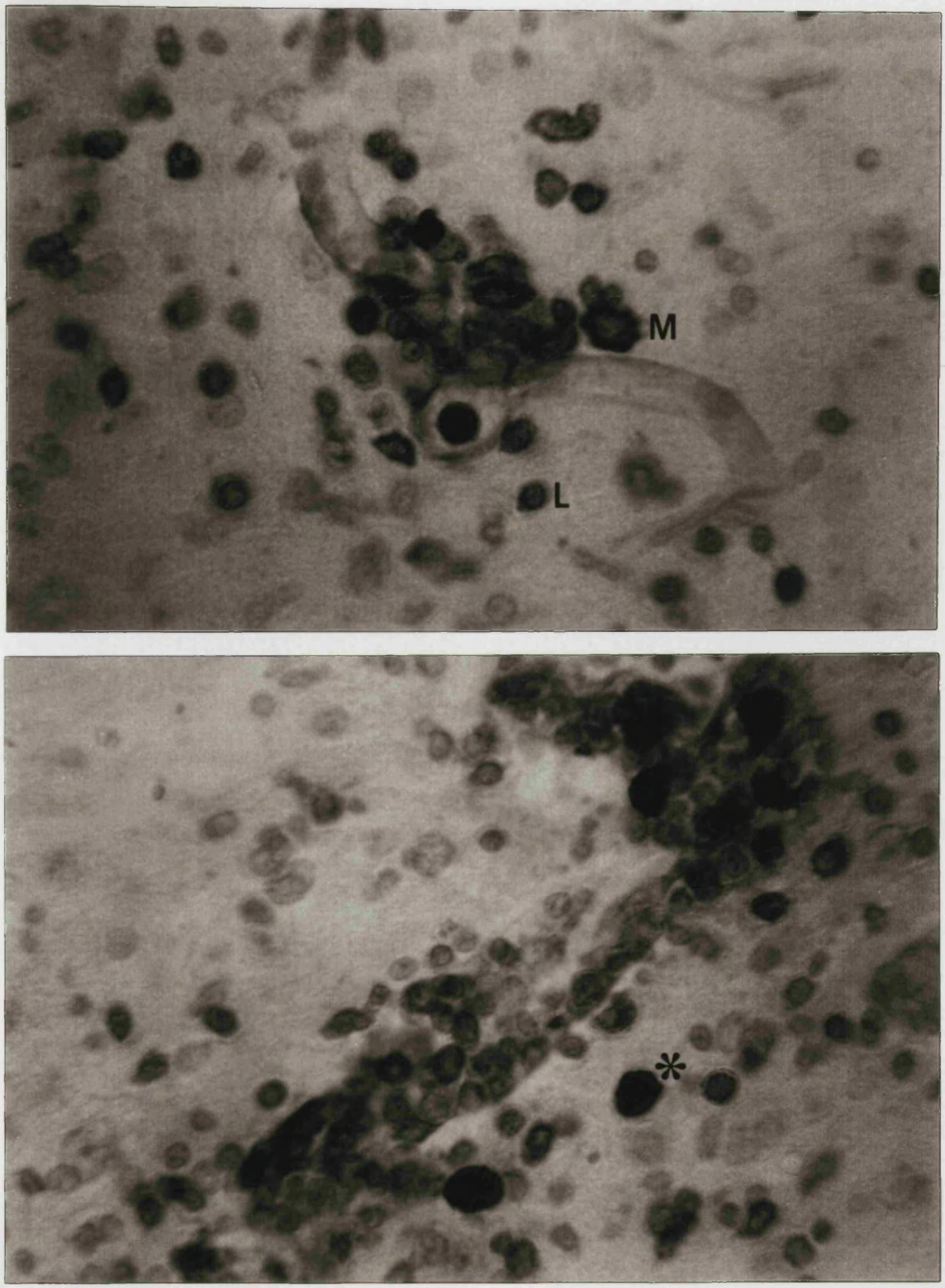


Figure 32. Immunolocalisation of lipocortin I in CNS lesions of EAE-diseased rats

Higher power views (x600) of two perivascular lesions illustrating lipocortin I immunostaining of macrophages (M), lymphocytes (L) and some very densely stained macrophage-like cells (*).

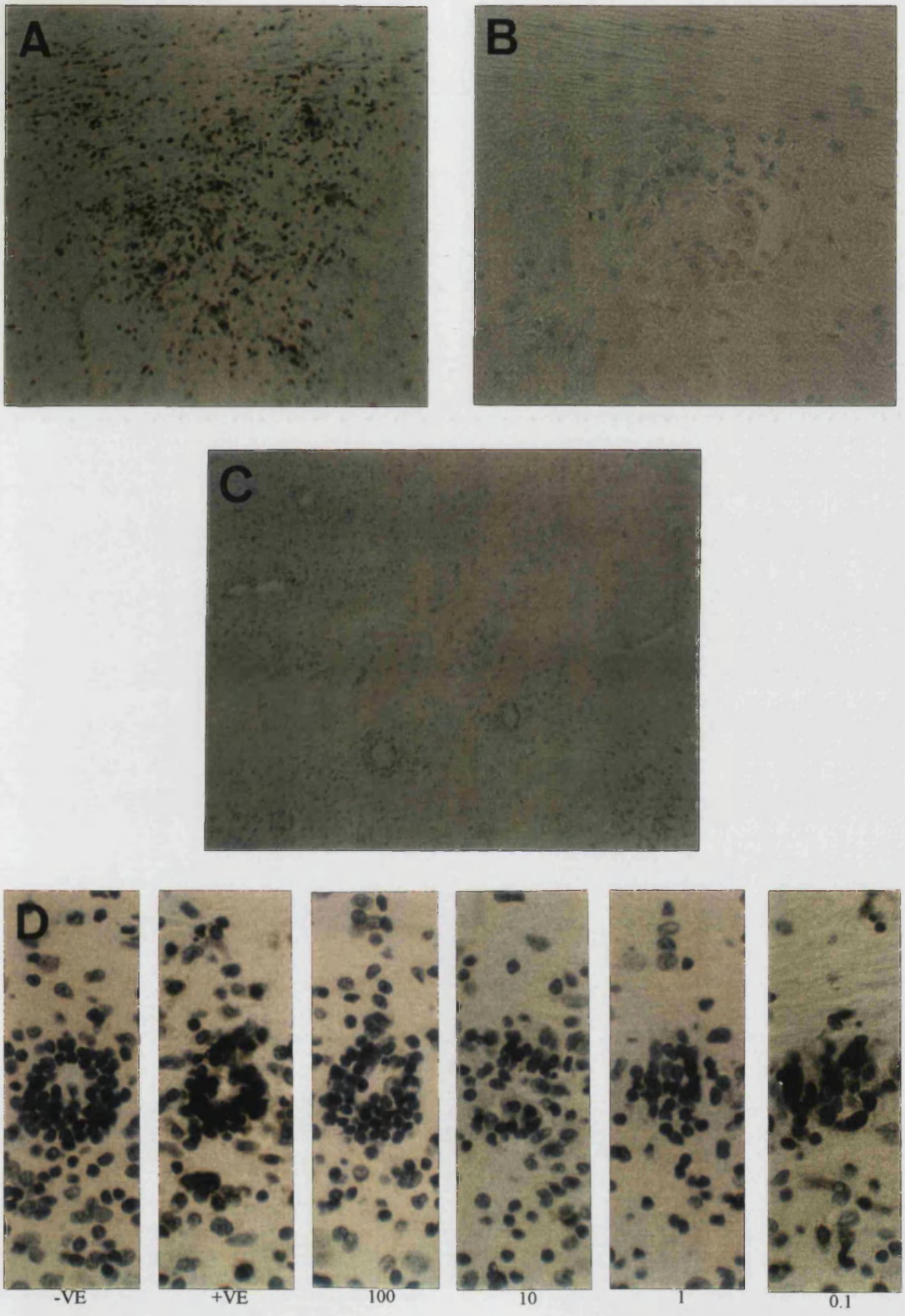


Figure 33. Control sections: EAE-diseased animals
Cervical spinal cord sections from EAE-diseased rats. (a) Positive control, immunostained for lipocortin I (x100). (b) Non-immune rabbit serum (x400). (c) Lipocortin I antiserum pre-incubated with 100 μg/ml rh lipocortin I (x100). (d) Serial sections through a single lesion demonstrating the effect of pre-adsorption of lipocortin I antiserum with various concentrations of rh lipocortin I. Figures below panels show concentration of lipocortin I in μg/ml. -VE, negative control, lipocortin antiserum omitted. +VE, positive control, lipocortin antiserum pre-incubated with PBS.

because much of the cell structure was masked by the intensity of staining, even on lightly stained sections.

Throughout the course of EAE there appeared to be no difference in the type or proportion of cells positive for lipocortin I, nor in the staining intensity of each cell-type. Furthermore, staining of blood vessels did not change during the disease and no specific staining of neurones, oligodendroglia or astrocytes was observed.

Control sections from EAE-diseased animals are shown in Figure 33. No staining was observed on sections where the lipocortin antisera was omitted or substituted with non-immune rabbit serum (Figure 33b). Figure 33c,d demonstrates the effect of pre-adsorption of the lipocortin antisera with various concentrations of rh lipocortin I. Pre-incubation of the antisera with 0.1 μg/ml lipocortin I did not appear to be sufficient to prevent staining, partial inhibition was observed with 1 and 10 μg/ml, but pre-incubation with 100 μg/ml was found to completely abolish staining of the lesions.

3.4.2.3 Serum Corticosterone

Corticosterone levels in the sera of rats used in the previous immunohistochemistry experiments are shown in Figure 34. Baseline serum corticosterone in normal animals was 39 ± 51 ng/ml (mean $\pm$ SD, n=9). In pre-diseased EAE-inoculated animals, corticosterone appeared slightly elevated at day 4 PI but had returned to baseline by day 7. However, clinically sick rats had serum corticosterone levels which were significantly higher than in normal controls and which progressively increased with the severity of symptoms, peaking at the paralysis stage at 276 ± 186 ng/ml ($n=7$, $P<0.01$). Following recovery, levels were reduced and although slightly elevated in comparison, they were not significantly different to normal animals. The serum corticosterone concentration of CFA-

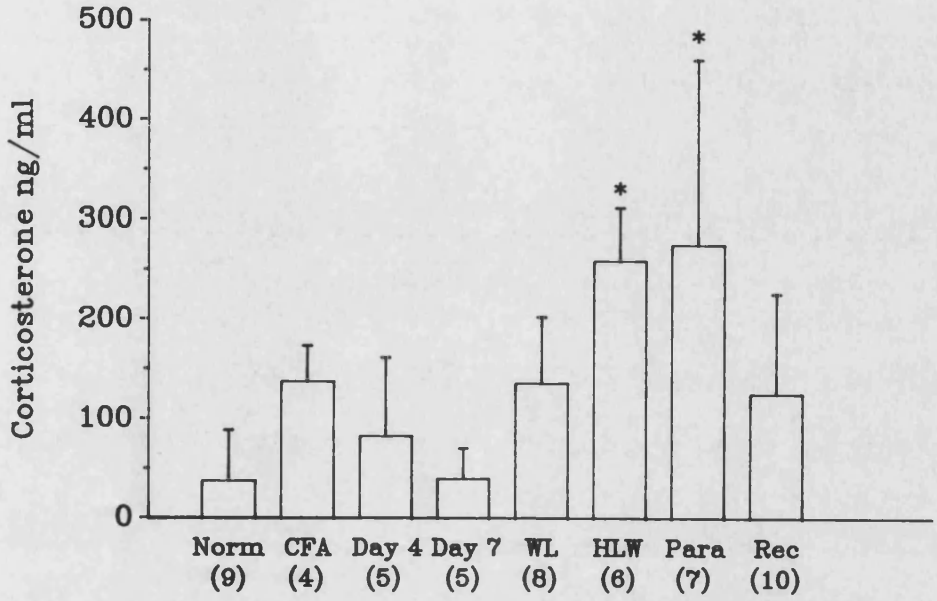


Figure 34. Corticosterone levels in the serum of normal and CFA-inoculated controls, and EAE-inoculated rats at various stages of the disease
Normal rats (Norm). CFA controls (CFA) were sampled 15 days post-inoculation. EAE-inoculated rats were sampled on day 4 or 7 post-inoculation or on the first day of exhibiting weight loss (WL), hind limb weakness (HLW), paralysis (Para) or recovery (Rec). Results are expressed as mean $\pm$ SD and are pooled from two separate experiments, the number of animals in each group is shown in parenthesis. Results were analysed using ANOVA and the significance of differences between group means determined using the Newman-Keuls procedure: * $P<0.01$ when compared to normal controls.

inoculated rats was 138 ± 36 ng/ml (n=4) and although this appeared to be higher than that of normal controls and somewhat lower than the value obtained for corresponding EAE-inoculated rats, neither difference reached statistical significance.

3.5 EFFECT OF MANIPULATING CIRCULATING STEROIDS ON LIPOCORTIN LEVELS IN THE CNS DURING EAE

Several methods were used to manipulate circulating corticosteroid levels in EAE-inoculated rats. In some experiments the plasma steroid concentration was raised by dosing animals with either the natural hormone corticosterone or with the synthetic steroid dexamethasone. In other experiments endogenous steroids were antagonised with the anti-glucocorticoid RU 38486 or abolished by removal of the source of natural steroids, the adrenals. To assess the effect of each treatment on CNS lipocortin levels, cervical spinal cords were removed and pooled samples Western blotted for lipocortins I, II and V using the polyclonal antisera 842, 774 and 890 respectively and the PAP Ni/Co detection method. On all blots the molecular mass of the major band was close to that reported for the appropriate lipocortin in the literature and the intensity of this band was measured using densitometry in order to provide a semi-quantitative estimate of the amount of immunoreactive lipocortin in each group. In addition, serum samples were collected for measurement of circulating corticosterone to determine the effectiveness of each treatment in influencing plasma steroid levels.

Previous experiments (see section 3.4.1) established that levels of immunoreactive lipocortin I in the CNS of EAE-inoculated rats changed during the course of the disease. Thus in experiments designed to compare lipocortins in the

CNS of drug treated and vehicle treated EAE-inoculated animals it was important to ensure that the two groups were well matched for stage of disease. Consequently, rats were usually dosed at the height of disease and were only used if they exhibited signs of complete or partial paralysis. Those displaying the same symptoms and course of disease were paired, with one receiving drug and the other vehicle. Unfortunately this degree of selectivity resulted in low sample numbers in some experiments because, although in each experiment 18-30 animals were inoculated for EAE, those which showed no symptoms, developed only a mild form of EAE, or died of the disease could not be included.

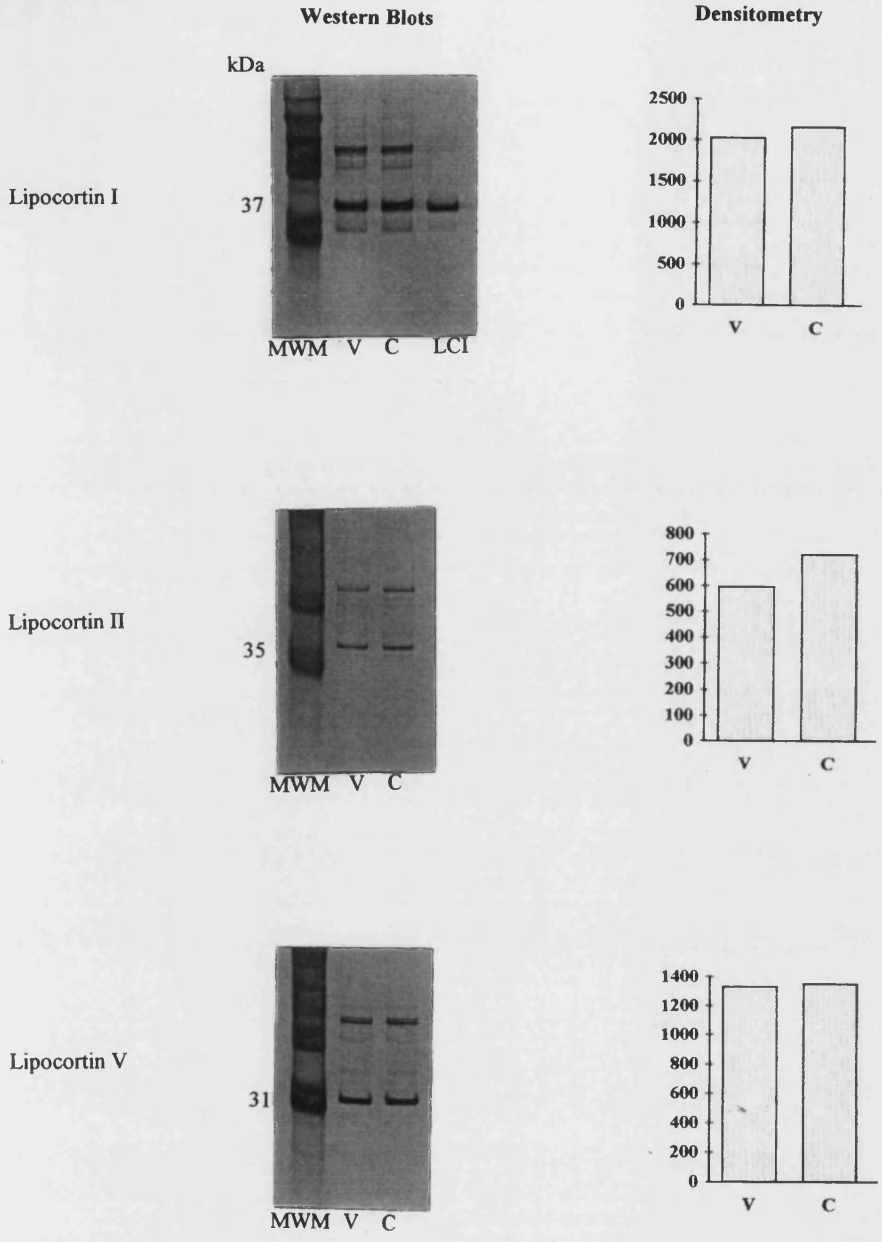
3.5.1 Corticosterone

Three separate experiments were performed to assess the effect of corticosterone administration on lipocortin levels in the CNS.

In the first experiment EAE-inoculated rats at the height of disease were dosed twice with 10 mg/kg corticosterone. Following treatment, clinical scores of corticosterone dosed animals seemed to be slightly decreased and there appeared to be some elevation in the serum corticosterone concentration in this group. However Western blotting and densitometry of cervical spinal cord samples revealed no difference between steroid and vehicle treated animals in the levels of immunoreactive lipocortins I, II and V (Figure 35).

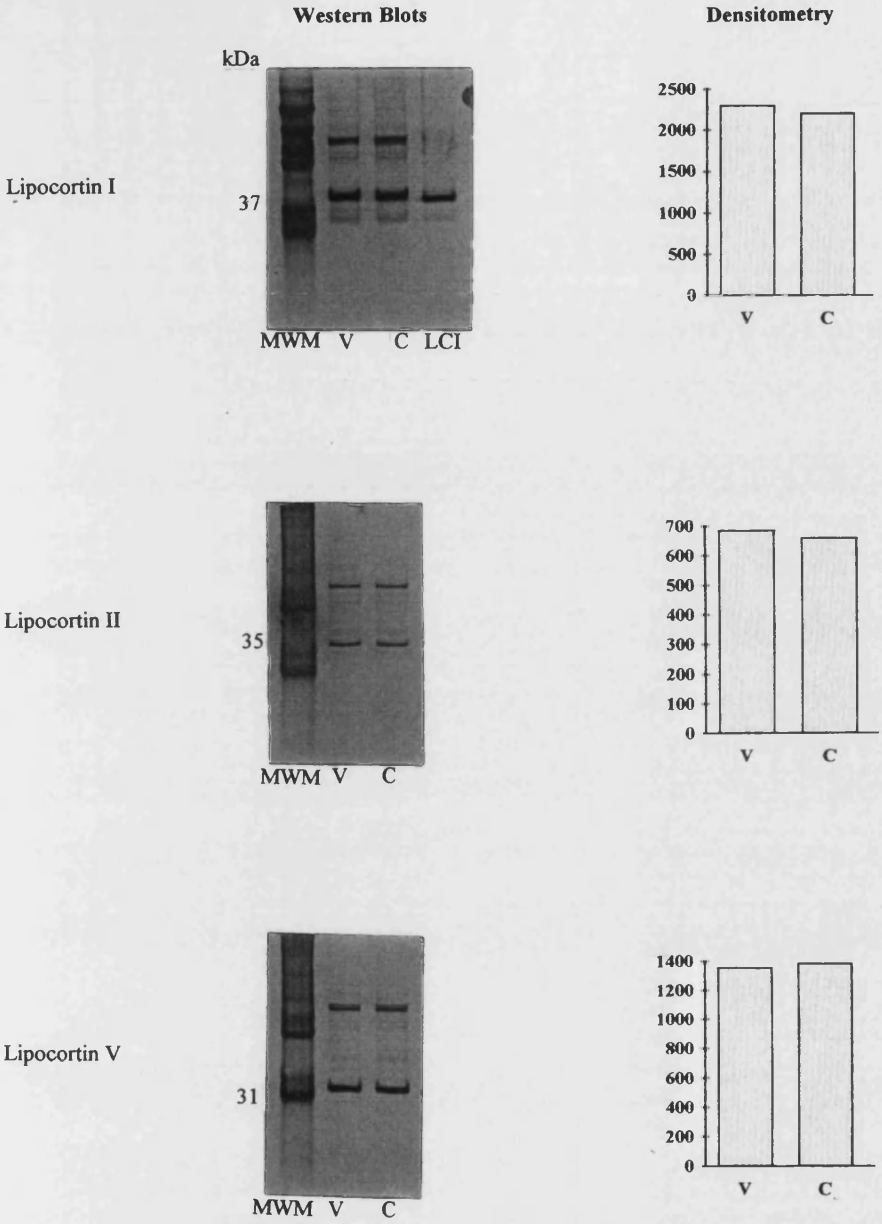
In the second study animals were dosed in the same manner as in the previous experiment but on the first day of complete recovery from paralytic symptoms. In this experiment treatment with corticosterone did not appear to cause an appreciable increase in circulating corticosterone levels and no difference in lipocortins I, II or V was detected (Figure 36).

In the third experiment corticosterone was again administered to rats at the height of disease but the dose was raised to 50 mg/kg and the number of doses



	n	Clinical Score		Serum Corticosterone ng/ml
		Before	After	
Vehicle	3	3.3 ± 0.6	3.3 ± 0.6	137 ± 69
Corticosterone	3	3.3 ± 0.6	1.7 ± 0.6	278 ± 219

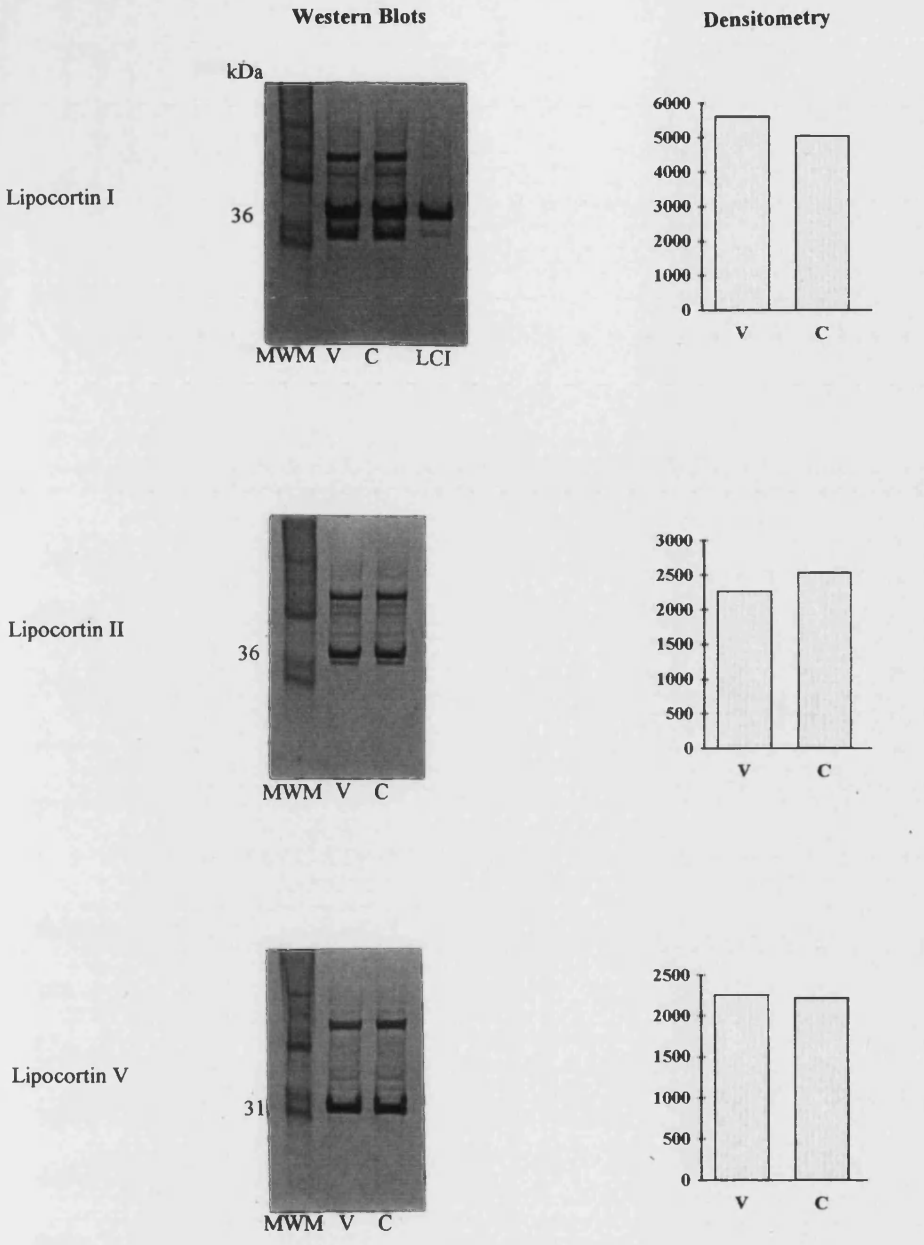
Figure 35. Effect of corticosterone (10 mg/kg) on lipocortins in the CNS of EAE-diseased rats
EAE-inoculated rats were dosed at the height of disease *sub. cut.* with 10 mg/kg corticosterone in 0.2 ml arachis oil twice, morning and afternoon, and then sampled the following morning. Vehicle controls received arachis oil only. Cervical spinal cord samples from vehicle (V) and corticosterone (C) treated animals were immunoblotted for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show densitometer values for the major band on each blot. Clinical scores (mean ± SD) were assessed before and after treatment. Serum corticosterone (mean ± SD) was measured at the end of the experiment.



	n	Serum Corticosterone ng/ml
Vehicle	3	86 ± 36
Corticosterone	3	105 ± 35

Figure 36. Effect of corticosterone (10 mg/kg) on lipocortins in the CNS of rats immediately after recovery from EAE

EAE-inoculated rats which had previously exhibited symptoms of complete or partial paralysis were dosed on the first day of complete recovery *sub. cut.* with 10 mg/kg corticosterone in 0.2 ml arachis oil twice, morning and afternoon and then sampled the following morning. Vehicle treated controls received arachis oil only. Cervical spinal cord samples from vehicle (V) and corticosterone (C) treated animals were immunoblotted for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show densitometer values for the major band on each blot. Serum corticosterone (mean ± SD) was measured at the end of the experiment.



	n	Clinical Score		Serum Corticosterone ng/ml
		Before	After	
Vehicle	5	3.6 ± 0.5	2.6 ± 1.1	185 ± 181
Corticosterone	5	3.2 ± 0.8	2.0 ± 1.4	672 ± 280

Figure 37. Effect of corticosterone (50 mg/kg) on lipocortins in the CNS of EAE-diseased rats
EAE-inoculated rats were dosed at the height of disease *sub. cut.* with 50 mg/kg corticosterone in 0.2 ml arachis oil three times, morning afternoon and the following morning, and were sampled two hours after the last dose. Vehicle treated controls received arachis oil only. Cervical spinal cord samples from vehicle (V) and corticosterone (C) treated animals were immunoblotted for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show densitometer values for the major band on each blot. Clinical scores (mean ± SD) were not significantly different before treatment (P= 0.445), and the change in score was not significantly different after treatment (P=0.665), Mann-Whitney U test. Serum corticosterone (mean ± SD) was significantly higher in the drug treated group, P<0.05, Mann-Whitney U test.

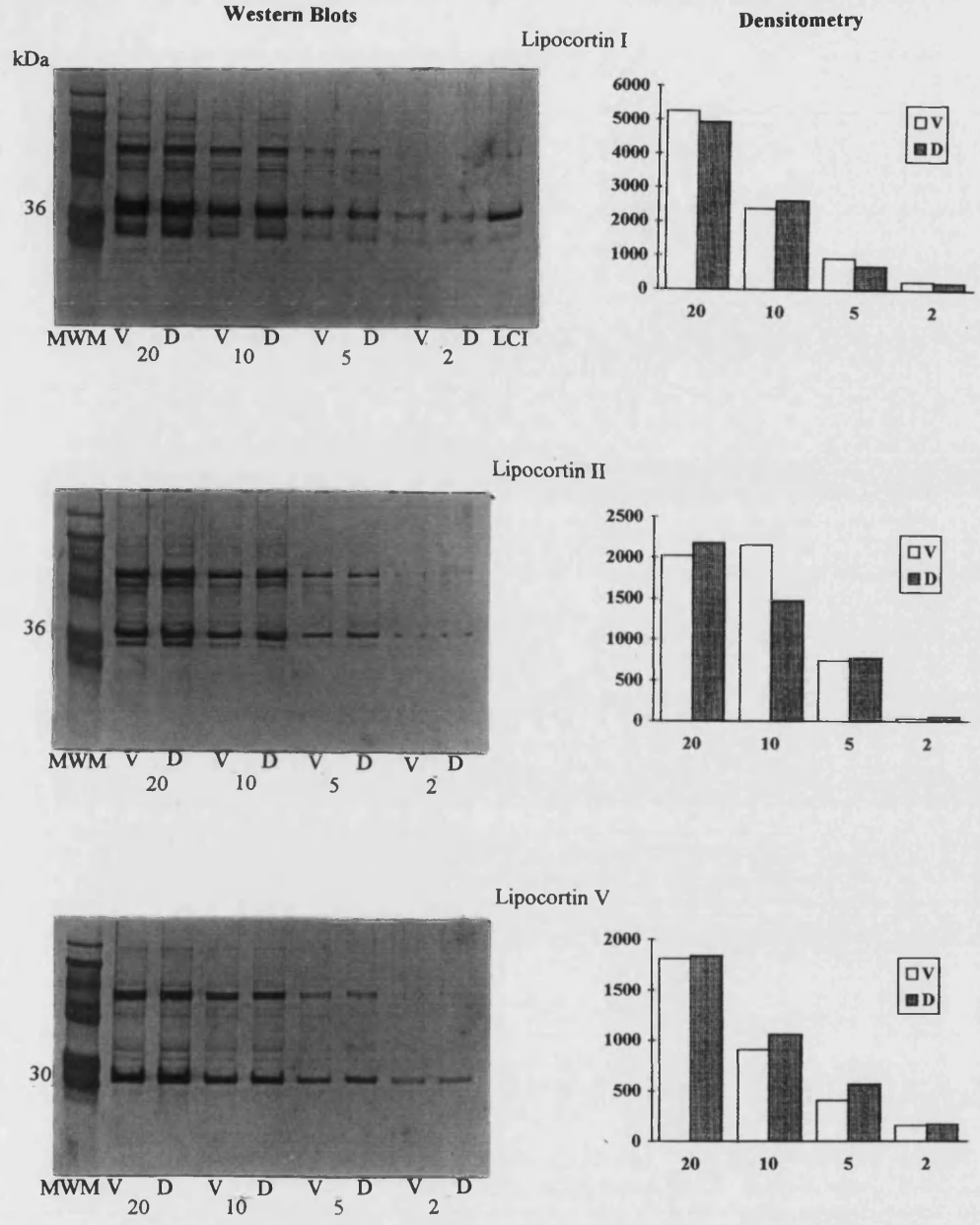
increased to three. This dosing regimen did not appear to influence the symptoms of EAE, since clinical scores were not significantly different before treatment and the change in scores was not significantly different after treatment. However, serum corticosterone was significantly elevated in the steroid treated group. Despite this, no difference in CNS lipocortin levels was observed (Figure 37).

3.5.2 Dexamethasone

The effect of dexamethasone sodium phosphate on lipocortins in the CNS during EAE was investigated in two separate experiments. Western blotting and densitometry are most sensitive at lower sample protein concentrations (see Materials and Methods, Figure 8), and to avoid small differences in lipocortin levels between vehicle and drug treated groups being masked by overloading, pooled CNS tissue samples were run at several different protein concentrations.

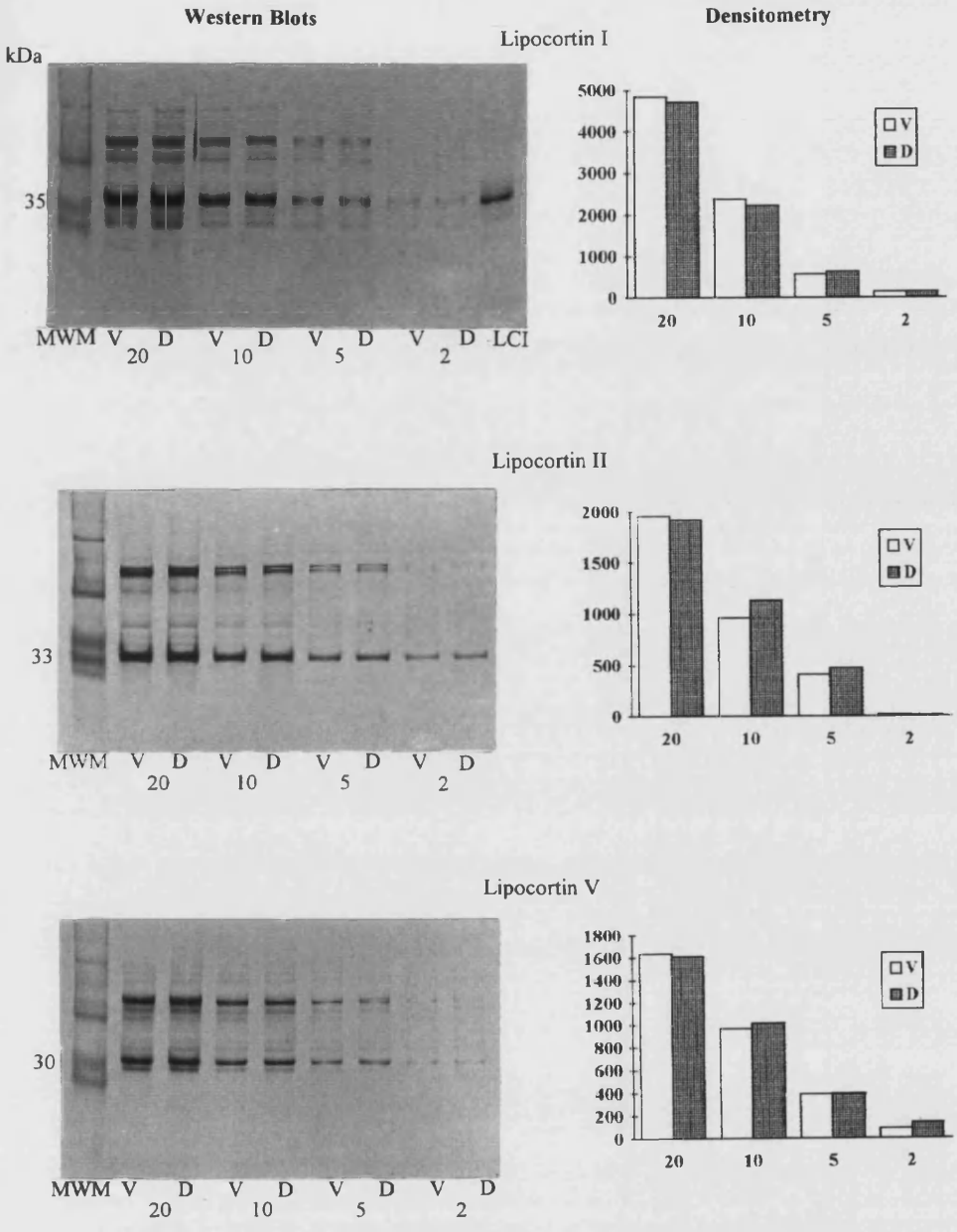
Results for the first experiment are shown in Figure 38. Animals were dosed three times at the height of disease with 0.5 mg/kg dexamethasone sodium phosphate. Treatment with dexamethasone did not reduce the clinical score. However mean serum corticosterone in the vehicle treated group was 125 ± 58 ng/ml, whereas in the dexamethasone treated group levels were below the limit of detection in three out of four animals. The fourth animal had a very high circulating corticosterone concentration, however this rat was very sick when sampled and appeared to be moribund. No difference was observed in the levels of lipocortins I, II or V in cervical spinal cords from vehicle and dexamethasone treated rats.

In a repeat experiment (Figure 39) the dose of dexamethasone was increased to 1 mg/kg. Again, dexamethasone treatment appeared to have no effect on neurological symptoms, but serum corticosterone was suppressed below the



	n	Clinical Score		Serum Corticosterone ng/ml
		Before	After	
Vehicle	3	4.0 ± 0.0	3.0 ± 1.0	125 ± 58
Dexamethasone	4	4.0 ± 0.0	4.0 ± 0.0	<*

Figure 38. Effect of dexamethasone (0.5 mg/kg) on lipocortins in the CNS of EAE-diseased rats
EAE-inoculated rats were dosed at the height of disease *sub. cut.* with 0.5 mg/kg dexamethasone sodium phosphate in PBS three times, morning, afternoon and the following morning and were sampled two hours after the last dose. Vehicle controls received PBS only. Blots show cervical spinal cord samples from vehicle (V) and dexamethasone (D) treated animals probed for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard; figures below each track indicate total protein loaded in µg. Histograms show densitometer values for the major band on each blot. Clinical scores were assessed before and after treatment. Serum corticosterone (mean ± SD) was below the limit of detection (25 ng/ml) in three out of four dexamethasone treated animals in the fourth it was 442 ng/ml (<*).



	n	Clinical Score		Serum Corticosterone ng/ml	
		Before	After	Inoculation	Sampling
Vehicle	7	3.6 ± 0.5	3.9 ± 0.4	110 ± 32	175 ± 111
Dexamethasone	7	3.5 ± 0.4	3.9 ± 0.4	117 ± 18	<

Figure 39. Effect of dexamethasone (1 mg/kg) on lipocortins in the CNS of EAE-diseased rats
EAE-inoculated rats were dosed at the height of disease *sub. cut.* with 1 mg/kg dexamethasone sodium phosphate three times, morning, afternoon and the following morning and sampled two hours after the last dose. Blots show cervical spinal cord samples from vehicle (V) and dexamethasone (D) treated animals probed for lipocortins I, II and V. MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard; values below each track indicate total protein loaded in µg. Histograms show densitometer values for the major band on each blot. Clinical scores were not significantly different before treatment (P=1.0) and the change in score was not significantly different after treatment (P=0.89), Mann-Whitney U test. Serum corticosterone (mean ± SD) was measured at inoculation and at the time of sampling and was below the limit of detection (25 ng/ml) in all dexamethasone treated animals (<). There was no significant difference in serum corticosterone between the two groups before treatment (P=0.710), but at the end of the experiment corticosterone levels were significantly reduced in the dexamethasone treated group, P<0.01 when compared to vehicle treated group at the time of sampling, Mann-Whitney U test; P<0.02 when compared to dexamethasone treated group at inoculation, Wilcoxon signed-rank test.

limit of detection in all dexamethasone dosed rats. No effect on CNS lipocortins was detected.

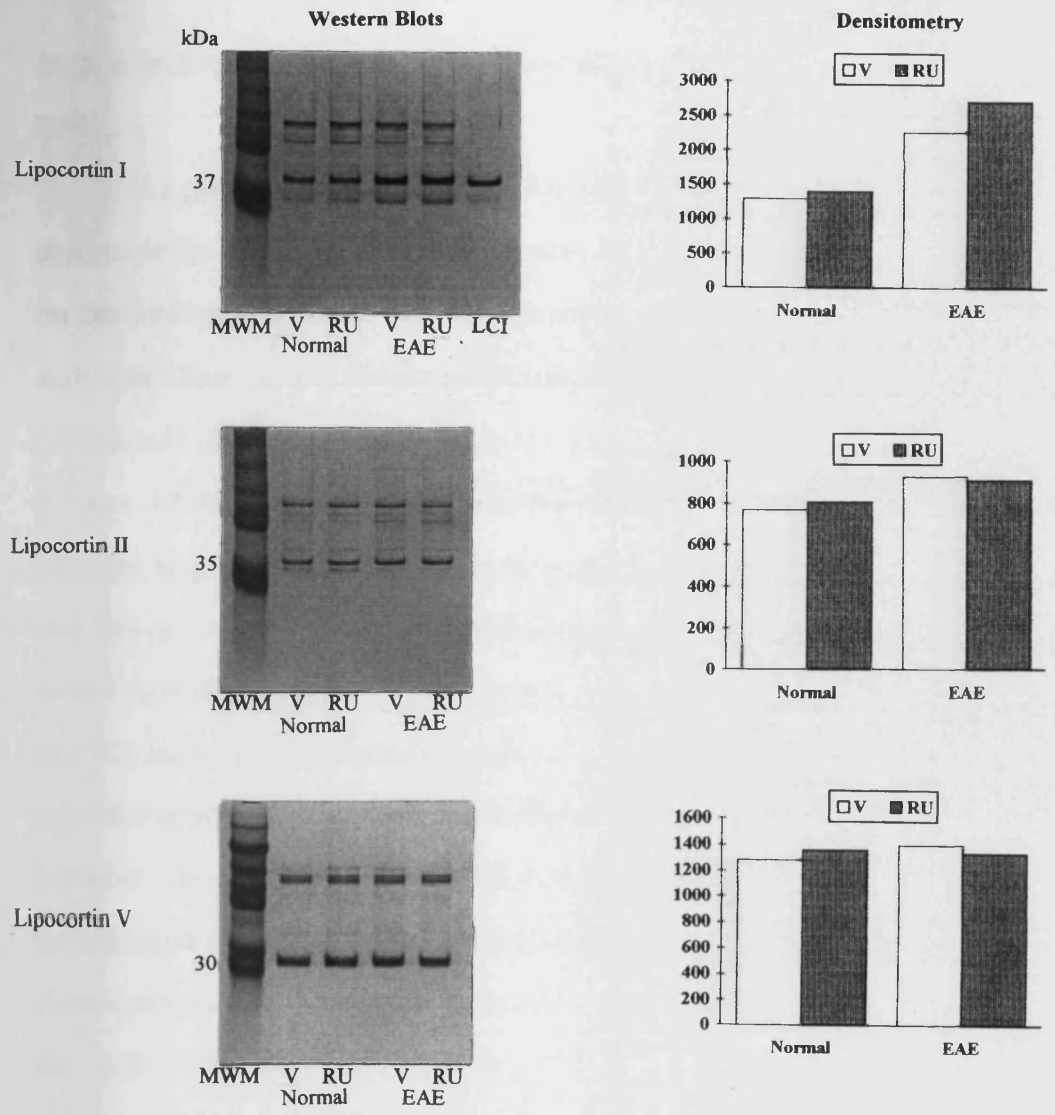
3.5.3 RU 38486

To assess the influence of endogenous corticosteroids on lipocortins in the CNS of EAE-diseased rats, animals were inoculated for EAE and dosed with the glucocorticoid antagonist RU 38486 on days 10-14 PI inclusive, starting prior to the onset of symptoms and the disease-related rise in serum corticosterone (see section 3.4.2.3 and Mackenzie *et al.* 1989, MacPhee *et al.* 1989). Control animals were inoculated for EAE but dosed with vehicle only. In addition the effect of RU 38486 on lipocortins in the CNS of normal animals was investigated.

Results are shown in Figure 40. The difference in clinical scores between drug and vehicle dosed EAE-inoculated animals was not statistically significant ($P=0.07$, Mann-Whitney U test). However at the time of sampling, rats in the RU 38486 treated group appeared to be moribund whereas those which had received vehicle seemed to be following a typical disease course. Corticosterone levels were not significantly different between drug and vehicle treated groups for either normal or EAE-inoculated animals, although there appeared to be a trend towards higher levels in the RU 38486 treated groups. Again, immunoreactive lipocortin I was found to be increased in cervical spinal cords from EAE-diseased animals compared to normal controls. However, treatment with RU 38486 did not appear to influence the levels of lipocortins I, II or V in CNS tissues from either normal or EAE-diseased rats.

3.5.4 Adrenalectomy

The influence of endogenous steroids on lipocortins in the CNS of EAE-diseased rats was also investigated by comparing lipocortin levels in cervical spinal



Group	Treatment	n	Clinical Score	Serum Corticosterone ng/ml
EAE	Vehicle	4	2.0 ± 1.6	218 ± 87
	RU 38486	4	4.0 ± 0.0	314 ± 139
Normal	Vehicle	4	-	29 ± 22
	RU 38486	4	-	62 ± 51

Figure 40. Effect of the glucocorticoid antagonist RU 38486 on lipocortins in the CNS of normal and EAE-inoculated rats

EAE-inoculated rats were dosed orally, twice daily on days 10-14 post-inoculation inclusive with 20 mg/kg RU 38486 in 1% carboxymethyl cellulose and 0.05% Tween 80. EAE-inoculated vehicle controls received vehicle only. Normal animals were dosed with RU 38486 or vehicle in the same manner for the same length of time. Cervical spinal cord samples were collected from RU 38486 (RU) or vehicle (V) treated animals the day after the last dose (day 15 post-inoculation for EAE-inoculated animals) and immunoblotted for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show the densitometer values for the major band on each blot. Clinical scores of EAE-inoculated animals were assessed at the time of sampling (day 15) and were not statistically different (P=0.07), Mann-Whitney U test. There was no significant difference in serum corticosterone between vehicle and RU 38486 treated groups for either normal (P=0.312), or EAE-inoculated (P=0.248) animals, Mann-Whitney U test.

cord samples taken from Adx, sham-operated and non-operated rats inoculated for EAE.

In a preliminary experiment six Adx and three sham-operated animals were immunised for EAE 7/12 days after surgery. Of the Adx rats, one was found dead on day 2 PI and one on day 15. The remaining animals succumbed to EAE very early (day 10 or 11) and developed an extremely severe form of the disease which progressed rapidly from no symptoms to complete paralysis over a period of about 8 hours. Of the sham-operated group, one animal showed no signs of disease and the other two developed very mild symptoms (flaccid tail on days 11-13 and 12-14) and then recovered, although one of these rats subsequently relapsed, exhibiting partial paralysis on day 23. Thus, whereas Adx rats were particularly susceptible to EAE, sham-operated animals appeared to be relatively resistant, and seemed to develop a delayed, milder form of the disease than that normally observed in the Lewis rat. Since previous studies had shown that lipocortin levels in the CNS may be influenced by stage of disease, it was originally intended to collect samples from sham-operated controls exhibiting the same symptoms as Adx animals. However the results of this initial experiment suggested that sham-operated rats were not the ideal control group for this type of study. In two subsequent experiments the following three groups were studied:

Adx - Rats were Adx and 11/12 days later inoculated for EAE. CNS samples were collected at the height of disease, approximately 10/11 days PI.

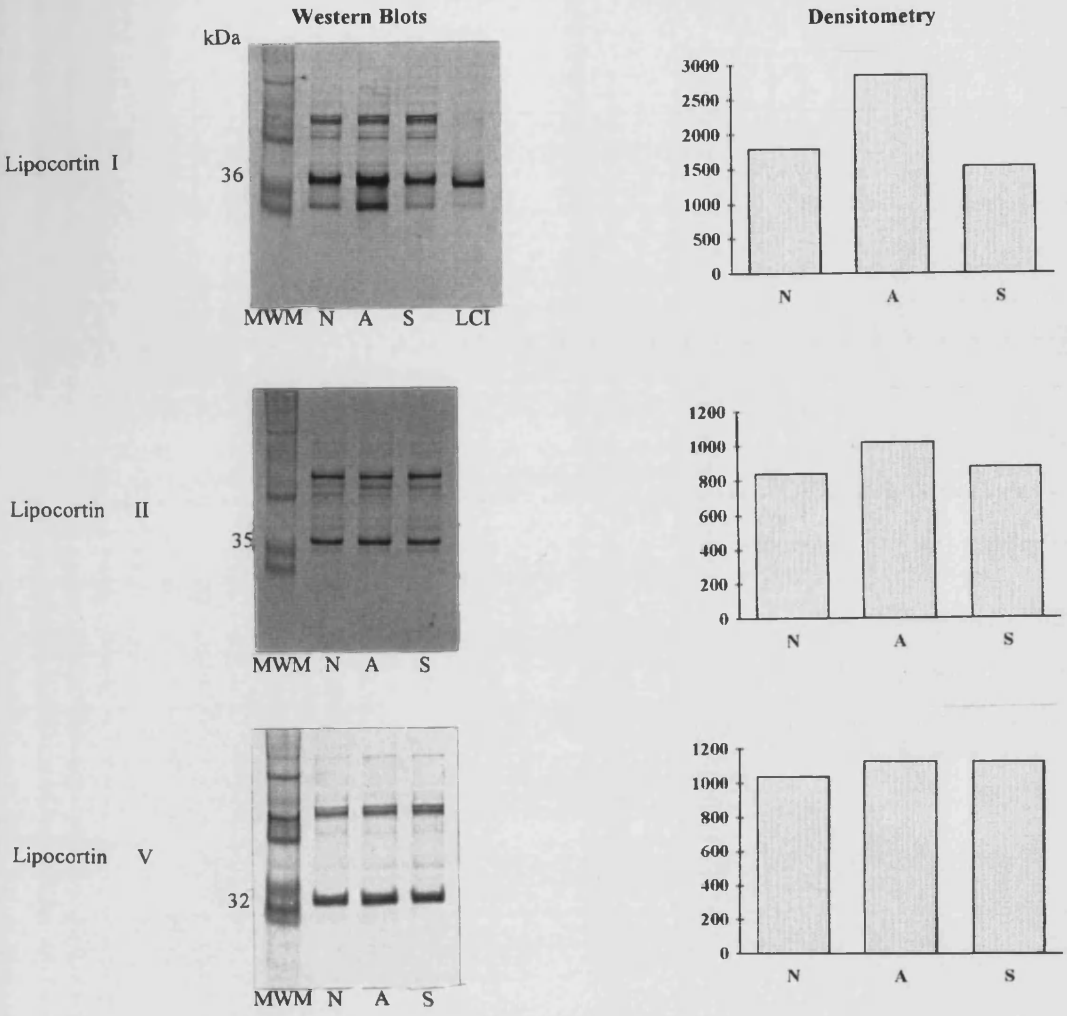
Sham-operated - Rats were sham operated and 11 days later inoculated for EAE. CNS samples were taken on day 10/11 PI providing time matched controls, although most of the animals showed no symptoms.

Non-operated - Normal, non-operated rats were inoculated for EAE. These animals were sampled at the height of disease, usually day 14, and were thus matched to the Adx group for stage of disease.

Serum samples for measurement of corticosterone were taken at inoculation and again when CNS samples were collected.

Two identical experiments were performed and similar results were observed in each. The majority of Adx rats developed a very severe form of EAE, characterised by an early onset and very rapid progression of symptoms. However several Adx rats which appeared healthy on day 9 or 10 were found dead the next morning and therefore could not be used. In addition, a few Adx animals did not develop signs of EAE until about day 14 and then exhibited a normal course of disease. CNS tissues from these animals were not used since it was assumed that resistance to EAE was due to the presence of residual adrenal tissue, subsequently these rats were found to have detectable levels of circulating corticosterone.

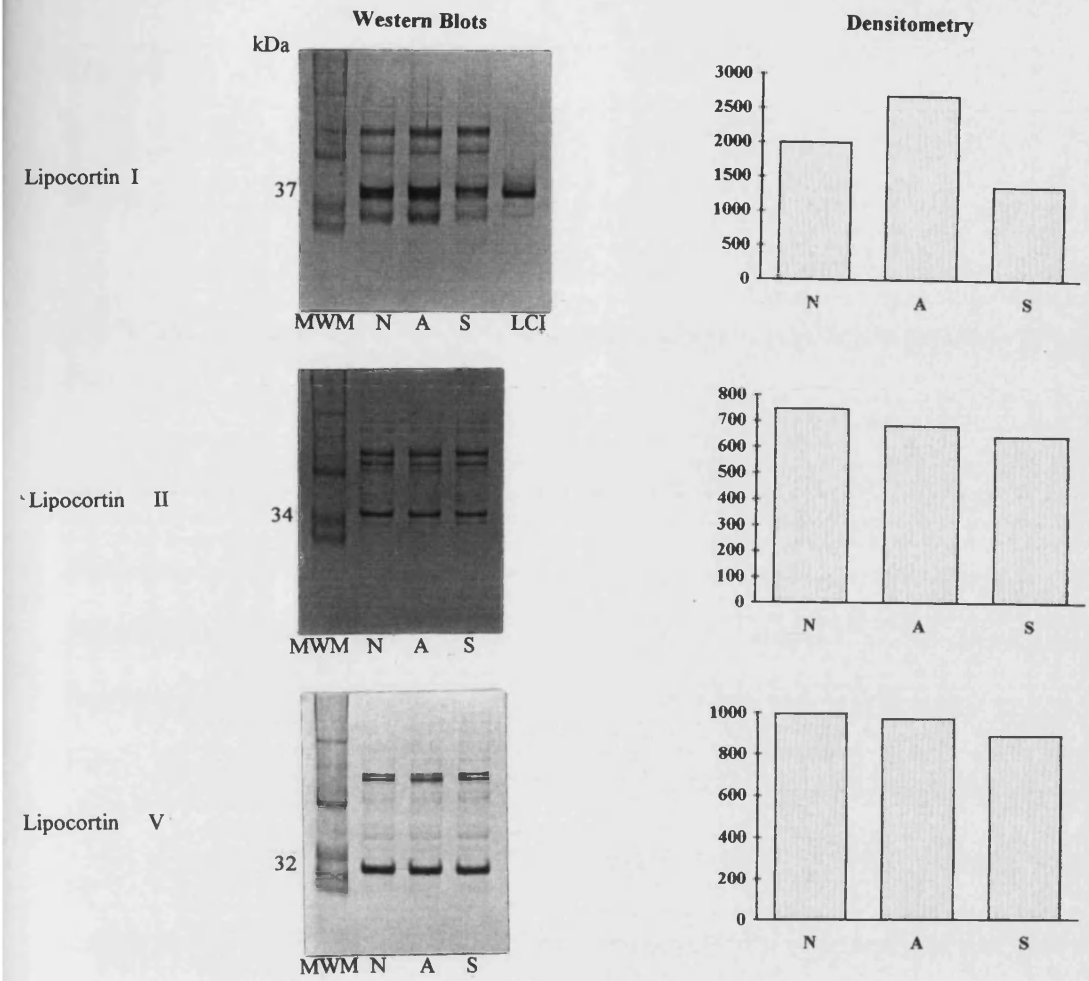
Results for the second experiment are shown in Figure 41. All Adx rats sampled, developed severe symptoms on day 10 PI whereas sham-operated controls killed on the same day showed no signs of disease. Non-operated rats were sampled at the height of disease and thus clinical scores were not significantly different to the Adx group. Serum corticosterone was below the limit of detection in all Adx rats used, both at the time of inoculation and at the end of the experiment and was thus significantly lower than in the other two groups at both time points. Differences between sham-operated and non-operated animals were not significant either at inoculation or at the time of sampling. Corticosterone levels in sham-operated animals appeared to decrease slightly during the experiment but the change was not statistically significant. Furthermore, circulating corticosterone in non-operated rats appeared to be increased at the height of disease but sample numbers were too low for valid statistical analysis.



Group	n	Day pi	Clinical Score	Serum Corticosterone ng/ml	
				Inoculation	Sampling
Non-operated	4	11/14	3.8 ± 0.5	177 ± 64	257 ± 92
Adrenalectomised	7	10	3.7 ± 0.5	<	<
Sham-operated	6	10	0.0 ± 0.0	195 ± 50	135 ± 59

Figure 41. Lipocortins in the CNS of adrenalectomised, sham-operated and non-operated rats inoculated for EAE: experiment II.

Adrenalectomised, sham-operated and non-operated rats were inoculated for EAE and sampled on days 10, 10 and 11/14 post-inoculation respectively. Blots show cervical spinal cord samples from non-operated (N), adrenalectomised (A) and sham-operated (S) animals probed for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show densitometer values for the major band on each blot. Clinical scores (mean ± SD) were assessed at the time of sampling and the mean score of the sham-operated group was significantly lower than that of adrenalectomised ($P<0.005$) and non-operated ($P<0.05$) animals, but there was no significant difference between the latter two groups ($P=0.913$), Mann-Whitney U test with the Bonferroni correction. Serum corticosterone (mean ± SD) was measured at inoculation and at the time of sampling, and was below the limit of detection (25 ng/ml) in all adrenalectomised animals at both time points (<). Corticosterone levels did not change significantly during the experiment in either sham-operated ($P=0.173$) or adrenalectomised rats, Wilcoxon signed-rank test. At inoculation there was no significant difference in serum corticosterone between sham-operated and non-operated animals ($P=0.522$) but levels in adrenalectomised animals were significantly lower than non-operated ($P<0.01$) and sham-operated ($P<0.005$) rats. At the time of sampling levels were still significantly lower in adrenalectomised rats than in non-operated ($P<0.01$) or sham-operated animals ($P<0.005$) and the difference between non-operated and sham-operated rats was not significant ($P=0.055$), Mann-Whitney U test with the Bonferroni correction.



Group	n	Day pi	Clinical Score	Serum Corticosterone ng/ml	
				Inoculation	Sampling
Non-operated	7	14	3.9 ± 0.4	95 ± 24	268 ± 152
Adrenalectomised	4	10/11	3.8 ± 0.5	<	<*
Sham-operated	6	10/11	0.3 ± 0.5	113 ± 19	64 ± 46

Figure 42. Lipocortins in the CNS of adrenalectomised, sham-operated and non-operated rats inoculated for EAE: experiment III.

Adrenalectomised, sham-operated and non-operated rats were inoculated for EAE and sampled on days 10/11, 10/11 and 14 post-inoculation respectively. Blots show cervical spinal cord samples from non-operated (N), adrenalectomised (A) and sham-operated (S) animals probed for lipocortins I, II and V: MWM, molecular mass markers; LCI, 10 ng rh lipocortin I standard. Histograms show densitometer values for the major band on each blot. Clinical scores (mean ± SD) were assessed at the time of sampling and the mean score of the sham-operated group was significantly lower than that of either adrenalectomised ($P<0.02$) or non-operated ($P<0.005$) animals, but there was no significant difference between the latter two groups ($P=0.718$), Mann-Whitney U test with the Bonferroni correction. Serum corticosterone (mean ± SD) was measured at inoculation and at the time of sampling, and was below the limit of detection (25 ng/ml) in all adrenalectomised rats at inoculation (<), although two animals had very low levels at the end of the experiment, approximately 10 and 23 ng/ml (<*). Within group comparisons showed a significant decrease in circulating corticosterone in sham-operated animals ($P<0.05$) and a significant elevation in non-operated rats ($P<0.02$) during the experiment, Wilcoxon signed-rank test. At inoculation there was no significant difference in serum corticosterone between sham-operated and non-operated animals ($P=0.253$), but levels were significantly lower in the adrenalectomised group than in either sham-operated ($P<0.02$) or non-operated rats ($P<0.02$). At the end of the experiment corticosterone levels in adrenalectomised animals were still significantly lower than in sham-operated ($P<0.05$) or non-operated ($P<0.02$) animals and levels in non-operated rats were significantly higher than in the sham-operated group ($P<0.05$), Mann-Whitney U test with the Bonferroni correction.

Lipocortin I immunoreactivity in the cervical spinal cord of Adx animals appeared to be elevated in comparison to sham-operated and non-operated controls. Immunoreactive lipocortins II and V seemed to be the same in all groups.

In the third experiment (Figure 42), Adx and non-operated animals were again sampled at the height of disease and statistical analysis of scores confirmed there was no significant difference between these groups. Two sham-operated controls showed mild signs of EAE at sampling (flaccid tail), but the mean score of this group was still significantly lower than that of the other two treatments. Serum corticosterone was below the limit of detection in all Adx animals at inoculation, although at the time of sampling two animals had very low circulating levels suggesting the existence of some residual adrenal tissue in these animals. However steroid levels did not appear to be sufficient to alter the course of the disease and at both time points the difference between adrenalectomised and other groups was statistically significant. At inoculation there was no significant difference in serum corticosterone between sham-operated and non-operated controls but by the end of the experiment levels in non-operated rats were significantly higher than in the sham-operated group. Moreover, during the course of the experiment there was a significant decrease in circulating corticosterone in sham-operated animals and a significant increase was observed in non-operated rats. As in the previous study, levels of immunoreactive lipocortin I in cervical spinal cord samples from Adx rats appeared to be higher than those of sham-operated and non-operated control animals. Furthermore, in this experiment levels in sham-operated rats appeared to be lower than those of non-operated animals. Again no difference in immunoreactive lipocortins II and V was detected.

DISCUSSION

4.1 LIPOCORTINS IN CSF

Studies on lipocortins in the CNS in MS and EAE were begun by investigating the lipocortin content of CSF. Cerebrospinal fluid is formed in the choroid plexus from plasma. It covers both the inner and outer surfaces of the brain and spinal cord and is continuous with the extracellular space of the tissue. There is free passage of solutes between the extracellular fluid and CSF across the ependymal lining of the ventricles and it is believed that the CSF acts as a sink into which the extracellular fluid and tissue metabolites drain. CSF is therefore considered to be useful for studying biochemical processes occurring within the CNS tissue (Walsh *et al.* 1983, Weller 1985). In inflammatory CNS diseases such as MS and EAE breakdown of the BBB results in the passage of blood-derived cells and plasma proteins into the CNS tissue, some of which may then pass into the CSF. Thus the protein content of the CSF in such conditions reflects not only production of proteins by endogenous and infiltrating cells within the brain, but also the entry of serum proteins into the CSF from blood vessels in the tissue (Walsh *et al.* 1983, Tourtellotte *et al.* 1988).

Initial studies were performed on cell-free CSF supernatants. When cell-free CSF samples from MS patients and controls were probed for lipocortins I, II, IV and V using Western blotting, all lipocortins were undetectable. Using the more sensitive ELISA technique extremely low levels of lipocortin I were observed in some of these samples, although the concentration was too close to the detection limit of the assay to be measured accurately. Likewise, investigation of the lipocortin content of cell-free CSF supernatants taken from normal and EAE-diseased rats also revealed that lipocortin I was below the limit of detection by Western blotting. This was despite observation of an increase in total protein in the CSF of animals with EAE.

Since lipocortins were virtually undetectable in cell-free CSF supernatants and some immunocytochemical and cell fractionation studies have suggested that lipocortins may be cytoplasmic or membrane associated proteins (Greenberg & Edelman 1983, Geisow *et al.* 1984, Glenney *et al.* 1987) the lipocortin content of whole CSF was investigated. Samples were lysed and treated with EDTA to allow measurement of total intracellular, extracellular and calcium-dependent membrane-bound protein.

Assessment of lipocortins in 39 samples of whole human CSF from patients with a variety of diseases revealed the presence of low levels of a 36 kDa lipocortin I-like protein in four samples by immunoblotting, which was confirmed in two of these using the lipocortin I ELISA. Two of the samples also contained low levels of a 31 kDa lipocortin I immunoreactive protein which is probably a degradation product of lipocortin I (Pepinsky *et al.* 1986, also see section 4.2 for fuller discussion of lower molecular mass bands). Small amounts of a lipocortin V-like protein were also detected in two samples. The CSF samples in which lipocortin-like proteins were detected were of diverse pathology. However two features common to all were the occurrence of very high protein levels and leukocyte counts. In fact, the four samples in which immunoreactive lipocortin I was detected had the highest number of leukocytes in the group. This observation together with the virtual absence of lipocortins in cell-free CSF (despite elevations in protein concentration) suggests that lipocortins in CSF are leukocyte associated.

The human CSF samples used in the present study were a random cross section of those obtained by Bath Royal United Hospital Pathology Laboratory. Unfortunately, only one of the samples was taken from a patient with MS. No lipocortins were detected in this CSF, but it cannot be ruled out that this sample was atypical of the situation in MS. However, it should be noted that all samples in which lipocortin-like proteins were detected contained at least 50 leukocytes per

mm^3 CSF. This is much higher than is generally found in MS where the total CSF leukocyte count is normal (< 5 per mm^3) in 66% of patients, < 15 per mm^3 in 95% and only rarely exceeds 50 per mm^3 (Walsh *et al.* 1983, Tourtellotte *et al.* 1988). Samples of whole CSF from patients with MS were not readily available, but since the amount of lipocortin in spinal fluid appears to be related to the leukocyte count, then in view of the range in leukocyte number normally found in the CSF in MS, it is unlikely that using the techniques in this study, significant quantities of lipocortins would have been detectable.

In studies on whole rat CSF, using the peroxidase conjugate immunoblotting technique, lipocortin I was undetectable in CSF from normal and CFA-inoculated rats and in EAE-inoculated animals during the induction phase, but low levels of a lipocortin I-like protein were seen in samples from diseased and convalescent animals. Experiments utilising the more sensitive PAP Ni/Co detection method revealed very low levels of immunoreactive lipocortin I in the spinal fluid of normal and CFA controls, and again a similar increase in this protein was observed in CSF from EAE-diseased animals, with a slight reduction following recovery. Proteins corresponding to the molecular mass of lipocortins II and V were undetectable in all rat spinal fluid samples but a band of approximately 51 kDa which was markedly increased in CSF from EAE-diseased animals was observed on blots probed for lipocortin II, the identity of this protein is not known (see section 4.2 for fuller discussion of higher molecular mass bands). Detection of lipocortins in CSF from animals in the disease and recovery phases of EAE coincided with an elevation in the CSF leukocyte count. As in human CSF, the highest levels were found in samples which had the greatest white blood cell counts, again suggesting that the lipocortin is associated with leukocytes.

To my knowledge there have been no previous studies on lipocortins in either human or animal CSF. The virtual absence of lipocortins from cell-free CSF

supernatants suggests that large amounts of lipocortin are not released from cells in the CNS either in normal or diseased states. Furthermore, since CSF is derived from plasma this result is consistent with the work of Goulding *et al.* (1990) who were unable to detect free lipocortin I in human plasma using the ELISA technique, although low levels of annexins I and V have been found in human plasma by other workers (Christmas *et al.* 1991).

In both the human and animal studies the lack of lipocortins in cell-free CSF supernatants and the presence of lipocortin-like proteins in lysate of whole CSF, but only in significant quantities when the leukocyte count is very high, all suggest that lipocortin in CSF is associated with infiltrating leukocytes. Moreover, the absence of a concordance between lipocortin levels and erythrocyte number indicates that the presence of lipocortin is not simply due to blood contamination.

The pleocytosis which was observed in CSF taken from animals with EAE is a characteristic feature of the disease. In Lewis rats inoculated for EAE the majority of cells in the CSF are lymphocytes and macrophages which are thought to be derived from inflammatory lesions, especially those in the meninges (Paterson 1978). Although cell types present in the human CSF samples were not determined, they are likely to be a mixture of lymphocytes, macrophages and polymorphonuclear leukocytes which have migrated from the blood into the CNS in varying proportions depending upon the nature of the disease. Although there have been no previous studies on lipocortins in CSF, the occurrence of lipocortin I in all these cell types (Pepinsky *et al.* 1986, Goulding *et al.* 1990) and the presence of lipocortin V in lymphocytes and monocytes (Pepinsky *et al.* 1988, Comera *et al.* 1989) is well documented.

In summary, results of studies on lipocortins in CSF revealed that very little lipocortin is present in rat or human CSF either under normal circumstances or in inflammatory CNS disease states. Furthermore, that which was present appeared

to be associated with leukocytes. Whole CSF from patients with MS was not available, but it is unlikely that significant amounts of lipocortin would have been detected due to the relatively low CSF leukocyte count found in the disease. In studies on EAE, although no problems were encountered in obtaining samples from normal and CFA-inoculated rats or EAE-inoculated animals during the induction phase, it proved very difficult to collect sufficient volumes of CSF from EAE-diseased rats. This appeared to be due to swelling of the brain tissue, resulting in occlusion of the cisterna magna and displacement of CSF. Levine *et al.* (1966) have observed this swelling and suggest it is caused by oedema. Due to the problems encountered in obtaining CSF and the very low levels of lipocortin detected in spinal fluid, studies on CSF were not continued. The apparent association of lipocortin I with cells in the CSF suggested that investigation of lipocortins in CNS tissue might prove more fruitful.

4.2 DETECTION OF LIPOCORTINS IN CNS TISSUE

Investigation of lipocortins I, II, IV and V in human CNS tissues by Western blotting revealed the presence of lipocortin immunoreactive material in both the grey and white matter of normal human CNS tissue. Similar proteins were detected in the spinal cord and cerebellum of normal Lewis rats. Generally, for both human and rodent tissue, the molecular mass of the principle band in each sample was close to that reported in the literature for the appropriate lipocortin, although in many cases they did not correspond exactly either with each other or with all literature reports.

It should be noted that the pre-stained molecular mass markers used in the present study to estimate the molecular mass of immunoreactive proteins only

appeared to be accurate to within ± 2 kDa. Furthermore, molecular masses reported for lipocortins in the literature tend to be very variable, with different values frequently reported for the same protein. This appears to be due, in part, to the tendency of lipocortins to give spurious size estimates on SDS-PAGE. For example Huang *et al.* (1987) have observed that in their hands the apparent size of lipocortin I by SDS-PAGE is approximately 35 kDa, which is nearly 4 kDa smaller than the 38.5 kDa its sequence indicates. In another interesting study Learmonth *et al.* (1992) identified two different forms of annexin V with apparent molecular masses of 33 and 37 kDa by SDS-PAGE, however complete sequence analysis of the two proteins revealed only two amino acid substitutions corresponding to a mass difference of 15 Da. Pepinsky and Sinclair (1986) have suggested that errors in estimation of the molecular mass of lipocortins may be due to the unusually charged amino acid composition of these proteins, since charged amino acids are known to affect electophoretic mobility in SDS-gels (Tung & Knight 1972).

When human CNS tissues were probed for lipocortin I using antibody 842, three main bands were detected with apparent molecular masses of approximately 37, 35 and 33 kDa. Studies on rat CNS tissues revealed a major band at approximately 38 kDa and a minor one at about 33 kDa. In each species the molecular mass of the largest peptide was identical to that reported in the literature for lipocortin I (Wallner *et al.* 1986, Pepinsky *et al.* 1988). Further evidence that these proteins may be lipocortin I was provided by their co-migration with rh lipocortin I standard. The detection of lipocortin I-like proteins in normal human and rat CNS tissues confirms the results of several other recent studies demonstrating the presence of immunoreactive lipocortin I in normal human (Johnson *et al.* 1989b) and rat (Smillie *et al.* 1989a, Smith *et al.* 1989b, Strijbos *et al.* 1991) CNS tissues.

The 35 and 33 kDa immunoreactive proteins detected in human CNS samples and the 33 kDa species found in rat tissues are very probably degradation products of lipocortin I. In early studies on lipocortin in rat peritoneal exudates, a significant proportion of the protein was found in a degraded form (Pepinsky *et al.* 1986). Subsequently lower molecular mass forms have been detected in human tissues: in BALF four separate proteins at 37, 36, 34 and 33 kDa were observed by Ambrose and Hunninghake (1990a) and a 34 kDa form by Smith *et al.* (1990b), a 35 kDa species has also been detected in placenta (Valentine-Braun *et al.* 1987) and in seminal plasma, a 32 kDa form lacking the first 29 amino acids has been found (Christmas *et al.* 1991). Furthermore in two recent studies on CNS tissue, 32 and 34 kDa forms of lipocortin I have been detected in rat brain homogenates (Gebicke-Haerter *et al.* 1991) and pig spinal cord and cortex (Regnouf *et al.* 1991) respectively.

In vitro studies have shown that the N-terminal domain of lipocortin I is highly susceptible to proteolysis and forms lacking different portions of this sequence can be generated through the action of exogenous proteases. Incubation with plasmin results in generation of 32 and 3 kDa cleavage products (Huang *et al.* 1987, Varticovski *et al.* 1988) whereas elastase yields several fragments of 33, 30, 27, 20, 18, 16 and 15 kDa (Huang *et al.* 1987). Calpain has been shown to cleave lipocortin I at Lys-26 to give a 34 kDa species, and cathepsin D at Try-12 to generate a 35.5 kDa form (Ando *et al.* 1989). In addition it has been observed that lipocortin I may be proteolysed by an endogenous A431 membrane protease (Chuah & Pallen 1989) and by conditioned media from BALF cells (Smith *et al.* 1990b).

Thus it is probable that the lower molecular mass species observed in the present study are breakdown products of lipocortin I, produced by enzymatic degradation of the protein. Although the medium used for homogenisation of CNS

samples contained the protease inhibitor PMSF, it is not known if these degradation products occur in significant amounts naturally or are an artefact generated during preparation of the tissue. Interestingly, a greater proportion of lipocortin I appeared to be in a degraded form in human CNS samples than in rat tissues. This might possibly be explained by the much longer death to freezing interval for human (mean 41 hrs) in comparison to rodent tissues (< 15 min).

An alternative explanation for the 35 kDa band observed in human tissue, is the possibility that this protein may be lipocortin III. Pepinsky *et al.* (1988) have reported that the lipocortin I antiserum 842, exhibits strong cross-reactivity with this protein, and the molecular mass of the band detected in this study is concordant with that reported for lipocortin III in the literature (Pepinsky *et al.* 1988). It should be noted however that this band was also detected by the anti-lipocortin I monoclonal antibody mAb 1B (Figure 20), which is not known to cross-react with other members of the lipocortin family (Pepinsky *et al.* 1990).

In both human and rat CNS tissues a number of weakly stained bands between 40 and 80 kDa were also observed. These were generally much fainter than the major bands, although in many samples fairly heavy staining of a 68 kDa immunoreactive protein was frequently detected. It is possible that this may arise from cross-reactivity of the antiserum with an unrelated protein, with a related protein such as the 68 kDa lipocortin VI, or with an as yet uncharacterised member of the annexin family. Alternatively, this protein could be a dimer of lipocortin I. A 68 kDa dimeric form of lipocortin I has been characterised in human placenta (Pepinsky *et al.* 1989) and a 74 kDa dimer in human epidermoid carcinoma cells (Ando *et al.* 1991). Cross-linkage appears to occur at Glu-18 in the amino terminal and can be induced following incubation of purified lipocortin with transglutaminase (Pepinsky *et al.* 1989, Ando *et al.* 1991). Recently, a dimeric

form of lipocortin I has been reported to be present in rat cerebral cortex (Pradel & Rendon 1993).

Investigation of lipocortin II in CNS tissues revealed only one band at approximately 37 kDa when rat tissues were probed using the polyclonal antiserum 774. However when human tissues were immunoblotted with the same antibody several bands were observed in the 30-40 kDa region, with the principle band at approximately 35 kDa. Using a different polyclonal antibody (Ab-1) which was raised against rh lipocortin II, only two immunoreactive species were detected in human tissue. A major one was found at 34 kDa and a minor one at 33 kDa, suggesting that the 35/34 kDa protein is probably lipocortin II, although unfortunately lipocortin II standard was not available for confirmation. The molecular masses of the main bands observed in human and rat CNS tissues are similar to those which have been reported for human lipocortin II, 38 kDa (Pepinsky *et al.* 1988), and human placental lipocortin II, 35 kDa (Huang *et al.* 1986). Several other studies have previously demonstrated the presence of immunoreactive lipocortin II in rat brain (Pepinsky *et al.* 1988, Smillie *et al.* 1989a) and cerebellum (Burgoyne *et al.* 1989). Although in contrast to the present study Reeves *et al.* (1992) were unable to detect expression of lipocortin II in normal adult human brain using immunohistochemistry.

Some of the lower molecular mass bands seen when human CNS tissues were probed for lipocortin II may well be degradation products of the protein. In studies on human placenta Huang *et al.* (1986) observed three species of lipocortin II with molecular masses of 34.5, 34 and 33 kDa and concluded that the smaller forms were generated by proteolysis. Lower molecular mass forms of lipocortin II have also been detected in CNS tissue: in rat cerebellum by Burgoyne *et al.* (1989) and in pig cortex and spinal cord by Regnouf *et al.* (1991). It should be noted however, that the polyclonal antiserum 774 also has quite a high titre of antibodies

that cross-react with lipocortin I (Pepinsky *et al.* 1988) and it is therefore possible that some of these proteins may be lipocortin I or its breakdown products.

In addition, a number of strongly stained higher molecular mass species were also seen, the main bands having molecular masses of approximately 61 and 54 kDa. Pepinsky *et al.* (1988) have also observed binding of the 774 antibody to higher molecular mass proteins in various tissues. The identity of these bands is not clear because, although lipocortin II is frequently found in association with the p11 protein, forming the heterotetramer calpactin I, at 90 kDa (Glenney & Tack 1985) the molecular mass of calpactin I is considerably higher than that of the proteins detected in the present study. Interestingly, Regnouf *et al.* (1991) have recently detected a 90 kDa annexin II immunoreactive protein which may be the heterotetramer in porcine spinal cord, although the protein appeared to be absent from brain cortex.

The antiserum raised against lipocortin IV (179) could not be used on rat CNS tissue because this antibody is known not to cross-react with the rat protein (J. L. Browning, personal communication). Investigation of lipocortin IV in human CNS tissues with this antibody revealed several immunoreactive bands, although there was only one in the 30-40 kDa region. At 40 kDa, the molecular mass of this band is slightly higher than that generally reported for lipocortin IV. Pepinsky *et al.* (1988) suggested a molecular mass of 35 kDa, but in most other studies on the protein, the molecular mass of lipocortin IV is quoted as 32.5 kDa (Klee 1988, Boustead *et al.* 1991, Massey *et al.* 1991). Due to the discrepancy in molecular mass it is not clear if the protein detected in human CNS tissue is lipocortin IV. The antiserum used for detection is thought to be specific for lipocortin IV and exhibits no cross-reactivity with known members of the lipocortin family (Pepinsky *et al.* 1988). However without access to lipocortin IV standard it was not possible to verify the identity of this protein.

There have been very few studies on lipocortin IV in CNS tissue. To my knowledge there have been no studies in the rat or in man, although lipocortin IV-like proteins have been detected in porcine and bovine brain (Woolgar *et al.* 1990, Regnouf *et al.* 1991).

When CNS tissues were probed for lipocortin V, only two principle bands were detected. In human tissue these were at 30 and 67 kDa, in rat samples the main band was at 35 kDa and a similar higher molecular mass band was also observed. Molecular masses reported for lipocortin V in the literature vary considerably. Pepinsky *et al.* (1988) described the molecular mass of rat and human lipocortin V as 35 kDa, but observed 32 and 33 kDa forms of the protein in bovine intestinal mucosa. Whereas a 32 kDa lipocortin V-like protein was detected in human peripheral blood mononuclear cells by Comera *et al.* (1989). Possible reasons for these discrepancies have been outlined earlier but they may also be due to tissue or species differences.

Several studies have previously reported the presence of lipocortin V-like protein in rat CNS tissue (Smillie *et al.* 1989a, Smith *et al.* 1989b, Donato *et al.* 1990, Pula *et al.* 1990, Giambanco *et al.* 1991) and the protein has also been detected in porcine (Woolgar *et al.* 1990) and bovine brain (Pula *et al.* 1990, Bianchi *et al.* 1992). In bovine brain Learmonth *et al.* (1992) found two isoforms of annexin V (CaBP33 & CaBP37) with apparent molecular masses of 33 and 37 kDa, although they reported that only the 33 kDa form was present in rat tissue which is concordant with the observation of a single band in the 30-40 kDa range in the present study. To my knowledge there have been no previous studies on lipocortin V in human CNS tissues.

Comparison of the levels of lipocortin-like proteins in normal human CNS tissues with samples from patients with MS revealed that immunoreactive lipocortins I, II, IV and V were significantly increased in white matter from MS

patients. Moreover, a further elevation in lipocortin immunoreactivity was observed in plaque tissue and immediately adjacent white matter such that, within the CNS of MS patients there appeared to be a gradient in the amount of each lipocortin, with the lowest levels in apparently unaffected white matter, an intermediate amount in tissue adjacent to a plaque, and the highest concentrations within plaque tissue.

An attempt was made to quantitate lipocortin I in human CNS tissue samples using the lipocortin I ELISA. This assay is a sandwich-type ELISA which was developed by Smith, Goulding and colleagues (Smith *et al.* 1990a) and utilises a monoclonal antibody (mAb 1B or 105) characterised by Pepinsky *et al.* (1990) and the polyclonal antiserum 842 (Pepinsky *et al.* 1988). It has a working range of 1-2000 ng/ml and has been used previously to measure lipocortin I in human BALF (Smith *et al.* 1990a) and human peripheral blood leukocytes (Goulding *et al.* 1990).

In a preliminary experiment samples were assayed at several different dilutions and it was noticed that when results were converted to ng lipocortin I /mg total protein, the amount of lipocortin I in any individual sample appeared to be different at each dilution, with the apparent lipocortin concentration increasing as the samples were diluted. This suggested that the CNS homogenates might be interfering with the assay.

To investigate whether this was the case, several representative samples were assayed, spiked with a known amount of rh lipocortin I. Ten nanograms of standard was added because this would approximately double the lipocortin concentration in normal CNS tissues, but both spiked and unspiked samples should still fall within the linear portion of the standard curve. Results showed that only a fraction of the standard added was measurable in the presence of CNS homogenates suggesting that either a constituent of the CNS tissue or a component

of the homogenisation medium, was preventing accurate measurement of lipocortin.

A further experiment was performed to find out if interference was caused by the homogenisation medium, the CNS tissue homogenate, or was influenced by the amount of lipocortin already present in the sample. Homogenisation medium alone appeared to have little effect on the measurement of lipocortin I. The presence of CNS tissue however, caused a marked reduction in the detection of lipocortin, even at relatively dilute protein concentrations, although the amount of lipocortin originally present in the sample did not appear to influence recovery.

The results of this study indicated that interference was due to a constituent of the CNS tissue rather than the homogenisation medium. To determine if this interfering substance might be a protein which was cross-reacting with one or both of the antibodies used in this assay, in the next experiment two identical blots containing representative CNS tissue samples were probed with each antibody. Both antibodies were found to bind only to lipocortin I and its putative breakdown products to any extent. This suggested that interference was not due to non-specific binding of the antibodies to other proteins present in the samples, and also demonstrates the specificity of the lipocortin I ELISA, when used on CNS tissue, for lipocortin I and cleavage products of the protein, since only proteins bound by both antibodies will be detected in the assay. Interestingly, Smith *et al.* (1990a) have suggested that this ELISA only detects the intact molecule and probably would not detect degradation products of lipocortin I. However, Pepinsky and co-workers who originally characterised the antibodies on which the assay is based have noted that the polyclonal antiserum 842 does react with lower molecular mass forms of lipocortin I (Pepinsky *et al.* 1988), furthermore, they report that the monoclonal antibody mAb 1B recognises a site between amino acids 30 and 55 in the lipocortin sequence and therefore recognises the commonly encountered N-

terminal clipped des-30 adduct (Pepinsky *et al.* 1990). Observations which are concordant with findings in the present study.

CNS tissue contains a high proportion of lipid which could potentially interfere with the assay by binding either to the lipocortin or to the monoclonal antibody. Thus a further study was performed to determine whether this might be the source of the problem and to investigate methods which might overcome this. An attempt to extract the lipid with chloroform resulted in removal of all the protein from the samples, although in it may have been possible to selectively extract lipid without affecting the protein using a less powerful chloroform/organic solvent mixture. High speed centrifugation was tried in an effort to remove liposomes from the samples, but this had no effect on measurement of lipocortin I. The addition of EGTA in order to chelate calcium and thus prevent calcium-dependent binding of lipocortin to phospholipids further reduced lipocortin detection.

From these results it is not clear if interference in the measurement of lipocortin in CNS samples is due to lipids or not. However, since no method was found which improved detection, attempts at the quantitation of lipocortin I in human CNS tissue by ELISA were abandoned.

For investigation of lipocortin levels in the rat CNS during EAE, the cervical spinal cord and cerebellum were studied because the former represents an area which is particularly susceptible to the development of lesions, and the latter a region which is less severely affected (Paterson 1978, Juhler 1988). Levels of lipocortin I-like protein were found to be elevated in cervical spinal cords taken from EAE-inoculated rats compared to normal controls. In pre-diseased animals a slight increase in lipocortin immunoreactivity was observed, but a much greater increase was found in cords from clinically sick rats which also corresponded with maximum cellular infiltration, levels then appeared to reduce following recovery

and regression of lesions. In the cerebellum a similar pattern was observed although changes in lipocortin I immunoreactivity were smaller in this region, which appeared to be a reflection of the lower number of lesions. Again greatest amounts were detected at the time when the lesion number was maximal, which in this area was during the recovery phase. In contrast levels of immunoreactive lipocortins II and V did not appear to alter in either area throughout the course of the disease.

Changes observed in lipocortin I-like protein during EAE are consistent with those found in the CNS of patients with MS. In EAE, lipocortin I immunoreactivity was increased in diseased tissue, and appeared to be proportional to the extent of lesioning. In MS, levels were also increased compared to normal controls, and the highest amounts were found within plaques. However immunoreactive lipocortins II and V were also found to be elevated in MS CNS tissue, but in EAE these proteins did not appear to change. This discrepancy could arise from inherent differences between the human disease and animal model, or possibly may reflect CNS regional differences since not exactly the same areas of the CNS were studied in each disease. An alternative explanation is that since differences in immunoreactive lipocortins II and V in MS CNS tissue were not of the same magnitude as those observed for lipocortin I, it may be that smaller changes in these proteins did occur during EAE but were not detected for the following reasons. 1) In studies on human CNS tissue lipocortins were detected using the PAP Ni/Co system, but when rat tissue was investigated this method had not yet been developed and the less sensitive peroxidase conjugate method was used. It should be noted however, that when the PAP Ni/Co technique was utilised in a later experiment investigating the effect of RU 38486 (section: 3.5.3) there appeared to be little difference in the levels of immunoreactive lipocortins II and V between normal and EAE-inoculated vehicle treated animals. 2) It is possible to

obtain large areas of relatively pure plaque tissue from patients with MS, whereas the tissue samples studied in EAE would have contained a large proportion of unaffected tissue. This would have the effect of diluting out any changes occurring principally within the lesions.

There have been no previous studies on lipocortins in the CNS in MS and EAE, although increased expression of lipocortins has been observed in other CNS diseases. Johnson *et al.* (1989ab) have found increased lipocortin I immunoreactivity in human brains exhibiting gliosis due to trauma, infarction or neurodegeneration and in many CNS glial tumours. Furthermore, expression of high levels of annexin II has been detected in human brains with high grade gliomas (Reeves *et al.* 1992). In another relevant study Relton *et al.* (1991) noted that following induction of cerebral ischaema in the rat, immunostaining for lipocortin I was markedly increased in infarcted brain regions. Moving away from the CNS, it is interesting to note that elevated levels of lipocortin I have also been found in diseased tissues in other inflammatory, steroid-responsive conditions such as lesional skin tissue in psoriasis (Bastian *et al.* 1991, Kitajima *et al.* 1991) and in BALF from patients with inflammatory lung diseases (Ambrose & Hunninghake 1990a, Smith *et al.* 1990ab).

Lipocortins have been proposed to act as "second messengers" of the anti-inflammatory effects of steroids. In the present study no corticosteroids were administered to animals with EAE and none of the patients with MS had received steroid therapy within the two years prior to post-mortem. However endogenous corticosteroids would of course be present circulating in the blood. It has been hypothesised by Munck and co-workers (1984) that one of the main physiological functions of circulatory glucocorticoids is to regulate inflammatory and immune mechanisms. Induction of an inflammatory/immune response causes stimulation of the hypothalamic-pituitary-adrenal (HPA) axis probably via the central action of

cytokines, and subsequently release of corticosteroids. Yet corticosteroids are known to possess profound immunosuppressive and anti-inflammatory properties. Munck suggests that normal basal steroid levels exert a constant modulatory effect on inflammatory and immune systems and that when stress due to infection or tissue damage causes steroid levels to rise they serve to switch off these systems in order to prevent overshoot and damage to the organism. Thus endogenous corticosteroids appear to mediate negative feedback on these processes, and presumably steroid therapy augments this natural mechanism. Since lipocortins have been proposed to be steroid-inducible molecules possessing anti-inflammatory (Flower 1988) and immunomodulatory (Hirata 1989) actions, it is conceivable that regulation of the immune system and inflammatory response by endogenous glucocorticoids might be mediated via the lipocortins. Thus it may be that lipocortins present in normal CNS tissue constitute the basal level required for normal immune and inflammatory regulation.

There is evidence that endogenous steroids play a role in both MS and EAE. In EAE, corticosteroid levels have been found to rise dramatically immediately prior to the onset of remission (Levine *et al.* 1980, Mackenzie *et al.* 1989, MacPhee *et al.* 1989). This surge in glucocorticoids appears to be responsible for self cure since if it is abolished by adrenalectomy animals do not recover (Levine *et al.* 1962), although this effect can be reversed by corticosteroid replacement (MacPhee *et al.* 1989). The increase in lipocortin I immunoreactivity detected in the CNS of animals with EAE happened at the same time as changes known to occur in serum corticosterone. Bringing together both Munck's work and the lipocortin theory it may be hypothesised that following induction of EAE, the release of cytokines and stress associated with the disease cause steroid levels to rise, this results in increased expression of lipocortin in the CNS which then mediates self cure.

Interestingly, a slightly elevated amount of immunoreactive lipocortin I was also found in CNS tissues from CFA-inoculated animals and small but significant rise in serum corticosterone has been observed in CFA-immunised rats on day 15 post-inoculation by Mackenzie *et al.* (1989). This may reflect non-specific stimulation of the immune system by the adjuvant and possibly the induction of sub-clinical adjuvant arthritis since injection of CFA into Lewis and other strains of rat is known to cause an experimental form of arthritis (Pearson 1956).

There is some evidence that dysfunction of the HPA axis occurs in patients with MS and that this may influence the course of the disease. However, the relationship between endogenous corticosteroids and disease activity is not nearly as well defined as in EAE. Some studies have shown that stress may affect onset of exacerbations in MS, but the nature of this influence is not clear as some workers have observed that stress appears to precipitate a relapse (Warren *et al.* 1982, Franklin *et al.* 1988, Grant *et al.* 1989), whereas others have found that stressful events seem to have a protective effect against disease exacerbation (Sibley *et al.* 1991, Nisipeanu & Korczyn 1993). Basal corticosteroid levels do not appear to be abnormal in MS. Although in one report elevated concentrations of plasma ACTH were detected (Allen *et al.* 1980) and in another basal cortisol values were observed to be increased in acutely relapsed patients (Millac *et al.* 1969), in many other studies unstimulated levels of urinary steroid metabolites and plasma cortisol have been found to be within the normal range (Garcia-Reyes *et al.* 1952, Teasdale *et al.* 1967, Brambilla *et al.* 1974, Maida & Summer 1979, Reder *et al.* 1987, Klapps *et al.* 1992). However, much evidence has accumulated which suggests that the post-stimulatory adrenocortical response may be defective in MS. Cortisol secretion following administration of ACTH has been observed to be markedly reduced in MS patients (Ketelaer & Delmotte 1972, Maida & Summer 1979) and the rise in cortisol and ACTH invoked by hypoglycaemia or other HPA

stimulants also appears to be impaired (Teasdale *et al.* 1967, Rinne 1968, Millac *et al.* 1969, Ketelaer & Delmotte 1972, Brambilla *et al.* 1974). In addition, a reduced response to the dexamethasone suppression test has been found in some patients (Reder *et al.* 1987). These studies suggest that adrenocortical secretory function may be inadequate in MS, although whether the deficiency occurs at the level of the adrenals, pituitary or hypothalamus in not clear. It has been postulated by Alexander *et al.* (1971) that such a defect may be inherent in MS patients and may contribute to the development of the disease.

The circulatory steroid levels of the MS patients from which CNS tissue samples were taken in the present study are not known. However in the light of the reports discussed above it may be postulated that the increased levels of lipocortins detected in the CNS of patients with MS may be due to induction of the proteins by endogenous corticosteroids and might represent an (unsuccessful?) attempt to overcome the chronic inflammation which is present in this disease. Moreover it is possible that lipocortin levels may be insufficient to suppress inflammatory and immune processes due to reduced efficiency of the HPA axis. The efficacy of steroid treatment may result from a further induction of lipocortins to a level which is sufficient to be effective in suppressing these mechanisms and thus alleviating symptoms of the disease.

One possible reason for failure to control the inflammatory process in MS may be that the lipocortin is inactivated. In studies on BALF, Smith and co-workers (1990b) observed that in healthy volunteers a high percentage of lipocortin I was in the native form, whereas a greater proportion was in a degraded N-terminal clipped form in BALF from patients with inflammatory lung disease. Since the N-terminal region appears to play an important role in regulating the function of lipocortins, they have suggested that enzymatic degradation may cause inactivation of the protein leading to uncontrolled inflammation. With respect to

the present study, it should be noted that statistical analysis of densitometer values obtained for the three bands detected in human CNS tissues from normal individuals and patients with MS, revealed no difference between any of the groups in the proportion of protein in each band, suggesting no difference in the degree of degradation.

In summary, elevated levels of immunoreactive lipocortins have been detected in CNS tissues from MS patients and the greatest amounts found within plaque tissue. In EAE, lipocortin I immunoreactivity was found to increase in CNS tissues during the course of the disease and this coincided with the appearance of inflammatory infiltrates in the CNS and with changes in serum corticosterone reported by other workers. Diseased CNS tissue in MS and EAE contains a great many lymphocytes and macrophages which are known to be a rich source of lipocortins. Thus the increased amount of lipocortins found in lesioned areas may be due to an influx of lipocortin containing cells and/or induction of the proteins in either endogenous or infiltrating cell types. However increases observed in normal appearing white matter in MS are more likely derived from indigenous cells because generally few infiltrating cells are found in white matter remote from plaque areas (Weller 1985). In the next stage of this study the distribution of lipocortins in CNS tissues from MS patients and EAE-diseased rats was investigated to determine the source of the increases in lipocortins observed in these diseases and in the case of EAE to clarify the relationship between lipocortin levels in the CNS and serum corticosterone.

4.3 DISTRIBUTION OF LIPOCORTIN I IN CNS TISSUES

The polyclonal antiserum 842 was used to investigate the distribution of lipocortin I in CNS tissues from MS patients and controls and in cervical spinal cord samples taken from normal and EAE-inoculated rats at various stages of the disease. The specificity of this antiserum was determined in previous Western blotting studies which showed that when used to probe human and rat CNS tissues this antibody reacts only with lipocortin I-like protein and putative breakdown products to any extent. It was not possible to study the distribution of lipocortins II, IV and V because the respective antibodies 774, 179 and 890 were all found to stain higher molecular mass bands with an intensity equal to that proposed to be the lipocortin protein and in the absence of lipocortin standards it would not be possible to determine the specificity of staining on CNS tissue sections by pre-adsorption experiments.

In normal human white matter lipocortin I immunoreactivity was found to be located primarily in blood vessel walls. A very similar distribution was observed in rat spinal cord where specific immunostaining for lipocortin I was seen in the walls of blood vessels and capillaries. A previous investigation of lipocortin I immunoreactivity in human CNS tissue by Johnson *et al.* (1989b) revealed very little immunostaining in the bulk of the white matter, although immunoreactive lipocortin I was detected in ependymal cells and a sub-population of astrocytes beneath the ependymal layer and surrounding the central canal of the spinal cord; areas which were not specifically investigated in the present study. However in a study on rat CNS tissue Strijbos *et al.* (1991) observed similar weak and somewhat patchy staining for lipocortin I in blood vessels throughout the brain. In addition, these workers found brain tanycytes, ependymal cells and certain varicose nerve fibres and neuronal cell bodies were also positive for lipocortin I. Interestingly,

immunoreactive lipocortin I was seen in the vascular endothelial cells of all other organs studied by this group. The presence of lipocortin I immunoreactivity in capillaries as well as larger blood vessels suggests that the protein may be associated with the endothelium and in support of this several studies have demonstrated the occurrence of lipocortin I in cultured endothelial cells (Hullin *et al.* 1989, Fujimoto *et al.* 1990, Patte *et al.* 1991, Raynal *et al.* 1992).

In normal CNS tissues very heavily stained cells were occasionally observed located within the lumen of blood vessels. Due to the intensity of staining, which tended to mask internal structure, many of these cells were difficult to identify although they are most likely to be polymorphonuclear leukocytes or monocytes since these circulatory blood cells have been shown to contain high levels of lipocortin I (Goulding *et al.* 1990, Comera *et al.* 1990). Furthermore, other workers have also found intravascular neutrophils and mononuclear cells to be amongst the most intensely stained cells present both in CNS and other tissues (Johnson *et al.* 1989ab, Fava *et al.* 1989).

In diseased CNS tissues lipocortin I immunostaining was markedly increased compared to the levels found in normal control samples. In CNS tissues from patients with MS, a slight elevation in lipocortin I immunoreactivity was observed in apparently uninvolved white matter remote from plaque areas, and a further substantial increase in immunostaining was found in actively demyelinating plaque tissue, confirming the results of previous Western blotting experiments. The increased staining in apparently normal MS white matter was due to the presence of scattered lipocortin I-positive astrocytes. In active plaques strong staining for lipocortin I was observed in the cell bodies and processes of large numbers of reactive astrocytes. In chronic plaques a dense network of immunostained astrocyte fibres was seen (data not shown). The accumulation of numerous reactive and hypertrophic astrocytes in the vicinity of active MS plaques

is a characteristic pathological feature of the disease. Moreover, in the centre of chronic plaques, fibrous processes from these cells form dense gliotic scars (Adams 1976, Weller 1985). In addition, scattered reactive astrocytes are frequently found throughout the parenchyma at some distance from the lesions (Allen & McKeown 1979, Newcombe *et al.* 1986).

The apparent absence of lipocortin I in quiescent glial cells in normal human CNS tissue and the detection of significant amounts of the protein in activated astrocytes in tissues from patients with MS suggests that expression of lipocortin I in astrocytes may be related to the level of cellular activation. Findings in the present study are in accordance with the work of Johnson and colleagues who also observed that lipocortin I was not expressed by histologically normal astrocytes in human CNS tissue, but found intense lipocortin I immunoreactivity in reactive astrocytes surrounding areas of infarction in cases of head trauma and intracerebral haemorrhage, and in scattered reactive astrocytes in patients with Alzheimer's disease (Johnson *et al.* 1989b). Furthermore, they have observed strong staining for lipocortin I in neoplastic cells of astrocytomas (Johnson *et al.* 1989a) and high levels of lipocortin I have also been detected in the C_6 glioma cell line (Huang *et al.* 1986).

Interestingly, in studies by Johnson *et al.* (1989ab) lipocortin I immunoreactivity was also observed in macrophages infiltrating CNS lesions. In contrast, in the present study although such cells were seen immunostained for lipocortin I within the walls of blood vessels, lipocortin I-positive macrophages were not found in the parenchyma, although staining of adjacent sections with the macrophage marker EBM 11 confirmed the presence of significant numbers of these cells within the tissue. Macrophages in the CNS of patients with MS may be derived either from blood monocytes or resident microglia (Adams *et al.* 1989), both of which are detected by EBM 11. Circulating monocytes are well

documented as being a rich source of lipocortin I (Comera *et al.* 1989,1990, Goulding *et al.* 1990, Browning *et al.* 1990) and the protein has recently been detected in cultured rat microglia (Gebicke-Haerter *et al.* 1991). The reason why macrophages present in the CNS in MS did not stain positive for lipocortin I is not known. The results observed appear to suggest that in MS, blood monocytes migrating into the CNS tissue cease to express lipocortin I and/or that activated microglia do not express the protein *in situ* in the CNS.

Investigation of the distribution of immunoreactive lipocortin I in cervical spinal cords from EAE-inoculated rats revealed that, prior to the onset of symptoms, the distribution and degree of staining was similar to that observed in normal animals. Using Western blotting a slight increase in lipocortin I immunoreactivity was detected in CNS tissues from pre-diseased rats and measurement of serum corticosterone suggested that circulating glucocorticoids were also slightly elevated. A small rise in corticosteroids during the induction phase of EAE has previously been observed by other workers (Mackenzie *et al.* 1989) and probably results from an acute reaction to the inoculum (Besedovsky *et al.* 1975, Schauenstein *et al.* 1987).

The emergence of clinical symptoms in rats immunised for EAE was accompanied by a marked increase in lipocortin I immunostaining which reached a maximum at the height of disease and then decreased following recovery, substantiating previous findings using Western blotting. Changes in the lipocortin I content of CNS tissues seemed to be proportional to the severity of symptoms and the number of lesions and also appeared to reflect serum corticosterone levels which were found to rise dramatically following the development of symptoms and then fell markedly after recovery. This pattern of adrenocortical response during the course of EAE has been reported in previous studies (Levine *et al.* 1980. Mackenzie *et al.* 1989, MacPhee *et al.* 1989).

In CNS tissues taken from CFA inoculated controls on day 15 post-inoculation the intensity and distribution of lipocortin I immunostaining appeared to be no different to that observed in normal rats and in pre-diseased EAE-inoculated animals, although a slight elevation in levels of immunoreactive lipocortin I had been detected by Western blotting. Assessment of serum corticosterone suggested that endogenous steroid levels might be slightly increased in these animals, a finding which has been reported in other studies (Mackenzie *et al.* 1989).

The source of changes in immunoreactive lipocortin I detected by Western blotting in CNS tissues from CFA controls and EAE-inoculated rats during the induction phase is not clear since on immunostained sections the degree and distribution of staining appeared to be the same as that found in normal animals. It is possible that smaller changes in lipocortin I immunoreactivity were not detectable using immunohistochemistry. An alternative explanation is that these slight increases were due to alterations in circulating levels of lipocortin. Goulding *et al.* (1990) have demonstrated induction of lipocortin I in blood cells following *in vivo* treatment with corticosteroids. Endogenous steroids appeared to be slightly raised in both CFA and pre-diseased rats and it is possible that this may have caused induction of lipocortin in systemic leukocytes. Since animals used in this study were exsanguinated but not perfused, an elevation in circulatory lipocortins may have been detected as an apparent increase in the CNS tissue by Western blotting. Such changes would not easily be observed on immunostained tissue sections.

The increased immunostaining in CNS tissues from EAE-diseased animals was found to be due to widespread staining of the lesions, but in contrast to findings in MS, extensive staining of infiltrating macrophages and lymphocytes was observed and astrocytes did not appear to stain positive for lipocortin I. In

addition, some very heavily stained cells were also seen in the vicinity of the lesions which appeared to be similar to some of those observed within the lumen of blood vessels in normal animals. These cells are probably blood derived monocytes/macrophages as the circulating cells known to contain the highest amounts of lipocortin I are monocytes and neutrophils (Comera *et al.* 1989,1990, Goulding *et al.* 1990), but polymorphonuclear leukocytes are rarely found invading the CNS in acute EAE in the Lewis rat (Paterson 1976, Shaw & Alvord 1984).

Although there have been no previous studies on lipocortins in the CNS in EAE, the association of lipocortin I with inflammatory cells is well established. The protein was originally isolated from macrophages (Flower 1988) and lower levels of lipocortin I protein and mRNA have been found in lymphocytes (Pepinsky *et al.* 1988, Bronnegard *et al.* 1988, Goulding *et al.* 1990). Furthermore in other immunohistochemical studies, lipocortin I immunoreactivity has been detected in resident tissue macrophages in various rat organs (Fava *et al.* 1989) and also in macrophages infiltrating lesions in human CNS tissue (Johnson *et al.* 1989ab).

In both MS and EAE increased immunostaining in CNS tissues was found to be localised mainly in lesioned areas. However, in MS lipocortin I immunoreactivity was located in reactive astrocytes but in EAE, positive staining was detected in infiltrating lymphocytes and macrophages. The reason for these differences in the distribution of lipocortin I, particularly the apparent absence of the protein in inflammatory cells invading the tissue parenchyma in MS, is not clear but may be due to differences between the pathology of the human condition and animal model. Leibowitz (1983) has suggested that much of the divergence between the two diseases is due to differences in disease duration and it is possible that this may explain differences seen in the expression of lipocortin I. In human CNS tissue lipocortin I immunoreactivity appeared to be present in reactive but not quiescent astrocytes, implying that expression of the protein in astroglia is

dependent upon the degree of cellular activation. A recent study by Reeves *et al.* (1992) suggests that the same is true of lipocortin II. Post-mortem studies on acute MS have shown that astrocytes do not become activated until about six weeks after commencement of an acute relapse (Adams *et al.* 1989). The duration of clinical disease in MS patients in the present study ranged from nine months to 49 years and it is unlikely that many, if any, of the plaques were less than six weeks old. Thus the plaque tissue studied would be expected to contain a considerable number of activated astrocytes.

In contrast to the situation in MS, Lewis rats were inoculated for an acute form of EAE. In this model visible lesions do not start to appear in the CNS until about day nine or ten post-inoculation, therefore when animals were sampled at the height of disease on day 14, lesions in the CNS were approximately 4-5 days old. It is possible that due to the short duration of disease there was insufficient time for astrocytes to become activated to express lipocortin I. In support of this suggestion, other studies have reported that reactive astrocytes are not present in acute EAE in the rat (Shaw & Alvord 1984). However some workers have detected increased immunostaining for GFAP (Smith *et al.* 1983, Cammer *et al.* 1990) suggesting that some activation of astrocytes does occur. Interestingly Gebicke-Haerter *et al.* (1991) observed that although lipocortin I was barely detectable in normal rat brain, cultured rat astrocytes rapidly accumulate high levels of the protein. Thus it may be that lipocortin I was not detected in astrocytes in acute EAE because the cells had not reached the required level of activation. It would therefore be very interesting to examine the distribution of the protein in a chronic relapsing model of EAE, where extensive astrogliosis and gliotic scar formation similar to that found in MS plaques, develop as the disease progresses (Smith & Eng 1987, Raine 1984).

It is difficult to rule out completely that some of the immunostaining observed in CNS tissue sections is not due to detection of lipocortin III. Cross-reactivity of lipocortin antibodies with other members of the lipocortin family is common because the core structures of these proteins are so highly homologous. Lipocortins I and III share 49% sequence identity and the 842 antiserum is known to have a high titre of antibodies that recognise rat lipocortin III (Pepinsky *et al.* 1988). It should be noted however that in rat CNS tissue all staining (except that of neuronal nuclei) was inhibited by pre-adsorption of the antiserum with rh lipocortin I. Furthermore, Comera *et al.* (1989) have reported that lipocortin III is absent from peripheral blood lymphocytes and monocytes, although to my knowledge there have been no studies which have investigated the presence of lipocortin III in astrocytes or endothelial cells.

As mentioned previously it was not possible to examine the distribution of lipocortins II, IV and V, however several other workers have recently investigated the immunolocalisation of lipocortins II and V in CNS and other tissues and it is interesting to note that the distribution of these proteins appears to be similar to that observed for lipocortin I. Like lipocortin I, lipocortin II also appears to be expressed by astroglial cells under certain circumstances. In a study on p36 (lipocortin II) in rat cerebellum Burgoyne *et al.* (1989) found the protein restricted to the cell processes of a sub-class of astrocytes. Furthermore, Reeves *et al.* (1992) found annexin II undetectable in normal adult human brain but observed high levels of expression in tumour tissue of glioblastoma multiforme and highly anaplastic astrocytomas, although not in astrocytomas of lower pathological grade. They also detected annexin II in glial scar tissue and in cell lines established from highly malignant glial tumours and suggest that expression of the protein may be dependent upon the degree of cellular activation. In addition, lipocortin II-like

protein has also been found in endothelial cells and macrophages in various rodent tissues (Gould *et al.* 1984).

A recent study on the distribution of lipocortin V in the rat, revealed high levels of the protein in the cell bodies and processes of both astrocytes and oligodendrocytes in the cerebellum and optic nerve. In addition lipocortin V was seen in capillary endothelial cells in many organs (Giambanco *et al.* 1991). Lipocortin V has also been detected in human peripheral blood lymphocytes and monocytes (Comera *et al.* 1989) and in rat peritoneal macrophages (Pepinsky *et al.* 1988). Furthermore, Patte *et al.* (1991) have reported the presence of immunoreactive proteins corresponding to lipocortins II and V in cultured human endothelial cells.

In the present study immunoreactive lipocortin I was detected in endothelial cells in normal CNS tissue and additionally in lymphocytes, macrophages and astrocytes in inflammatory CNS disease. Studies by other workers indicate that lipocortins II and V may also be present in these cell-types. The precise function of the various members of the lipocortin family has not been determined, but the occurrence of these proteins in endothelial cells, astroglia, macrophages and lymphocytes in MS and EAE suggests a physiological role for lipocortins in the functioning of these cells and by inference in the pathological processes in which they are involved.

Lymphocytes and macrophages are key effector cells of the immune response and appear to play a fundamental role in the pathology of MS and EAE. Many studies have shown that cytokines, enzymes, eicosanoids and other mediators released by these cells can initiate and perpetuate inflammatory and immune responses (Nathan *et al.* 1982, Geczy 1984, Davis 1985). Macrophages also appear to be responsible for demyelination (Wisniewski 1977, Prineas & Connell 1978) which may be mediated via the action of PLA_2 (Woelk *et al.*

1974,1976, Trotter & Smith 1984). In addition, these cells are probably the major source of the increased levels of PLA_2-derived eicosanoid inflammatory mediators found in the CNS in MS and EAE (Humes *et al.* 1977, Rosnowska *et al.* 1981, Bolton *et al.* 1984ab).

Mononuclear cells obviously play a pivotal role in inflammatory CNS disease and potentially the lipocortin they contain could influence many of the factors outlined above. Both lymphocytes and macrophages have glucocorticoid receptors (Lippman & Barr 1977, Werb *et al.* 1978) and many aspects of their function are suppressed by steroids (Fauci 1979). Recently it has been shown that lipocortins can duplicate many of the anti-inflammatory (Flower 1988) and immunomodulatory (Hirata 1989) effects of corticosteroids. In particular, rh lipocortin I has been demonstrated to inhibit migration of leukocytes into inflammatory lesions (Errasfa & Russo-Marie 1989), and to block the generation of superoxide (Maridonneau-Parini *et al.* 1989) and prostaglandins by macrophages (Cirino & Flower 1987b). Interestingly, Goulding and Guyre (1992) have recently discovered specific binding sites for lipocortin I on the surface of monocytes which they claim are receptors via which lipocortin I may influence the inflammatory actions of these cells.

Astrocytes perform several important functions in the CNS, they provide structural and metabolic support for neurones and appear to be involved in maintaining the integrity of the BBB. In addition, these cells play a key role in repair following damage to the CNS tissue and there is also evidence that astroglia have immune functions in the CNS. IL-1 and other cytokines have been shown to be mitogenic for astrocytes both *in vitro* and *in vivo* (Giulian & Lachman 1985, Giulian *et al.* 1988, Selmaj *et al.* 1990). Thus products from inflammatory cells may stimulate astrocyte proliferation and contribute to the reactive gliosis that is a prominent feature of MS plaques. Furthermore, astrocytes can be induced to

express Class II MHC antigenic determinants on their surface and expression of these antigens by astroglia has been detected in the CNS in MS and EAE (Traugott *et al.* 1985, Hickey *et al.* 1985, Lee *et al.* 1990). *In vitro* studies have demonstrated that astrocytes can present MBP to encephalitogenic T cells (Fontana *et al.* 1984), suggesting that these cells may function as antigen presenting cells *in vivo* and thereby up regulate the T lymphocyte response. There is also evidence that astrocytes may modulate immune reactivity in the CNS by releasing cytokines and other mediators, as cultured astrocytes have been found to produce IL-1 (Fontana *et al.* 1982), IL-3 and IL-6 (Frei & Fontana 1989), IFNs (Borgeson *et al.* 1989), tumour necrosis factor-α (Chung & Benveniste 1990), PGs (Fontana *et al.* 1982, Keller *et al.* 1985) and complement components (Levi-Strauss & Mallat 1987). Moreover, astroglia appear to release factors that can suppress T cell proliferation and/or function and thus these cells may also mediate immunosuppression in the CNS (Borgeson *et al.* 1989, Matsumoto *et al.* 1993).

Steroids have been shown to inhibit astrocyte growth (Kniss & Burry 1985, Grasso 1976, Armelin & Armelin 1983) and to influence other aspects of astroglial function *in vitro* (De Vellis *et al.* 1986). Furthermore, these compounds are known to have suppressive effects on the release of many of the immune and inflammatory mediators described above, and have been demonstrated to block production of cytokines (Nishida *et al.* 1989) and eicosanoids (Gebicke-Haerter *et al.* 1991, Brenner *et al.* 1992) by astrocytes in culture. Presumably some of these steroid effects on astrocytes may be mediated by lipocortins.

It is also interesting to note that lipocortins may be involved in the regulation of astrocyte proliferation. One of the mediators proposed to induce astrogliosis is epidermal growth factor (EGF). EGF is mitogenic for astrocytes *in vitro* (Leutz & Schachner 1981, Simpson *et al.* 1982), furthermore EGF receptors are found on astrocytes on the CNS (Simpson *et al.* 1982) and enhanced expression of the gene

encoding the EGF receptor/kinase has been observed in astrocytic neoplasms (Libermann *et al.* 1984). p35 (lipocortin I) is known to be the major physiologic substrate for the EGF receptor/kinase (Fava & Cohen 1984, Pepinsky & Sinclair 1986, Huang *et al.* 1986), and may therefore be important in the signal transduction pathway of the EGF receptor, and thus in the regulation of astrocyte proliferation, astrogliosis and tissue repair. This mechanism may offer an alternative explanation for the increased expression of lipocortin I in reactive astrocytes in MS.

The presence of lipocortin I in CNS endothelial cells is particularly interesting since these cells constitute a major structural component of the BBB. Increased BBB permeability and CNS oedema are well defined features of MS and EAE which may be responsible for inhibiting neuronal conduction and thus causing the physical symptoms of these diseases (Sears *et al.* 1978, Paterson 1982, Simmons *et al.* 1982). The anti-oedematous properties of corticosteroids are well known and they are widely used for the clinical treatment of brain oedema (Reulen *et al.* 1972). Studies using computed tomography and magnetic resonance imaging suggest that corticosteroid therapy can reduce BBB abnormalities and oedema in MS (Sears *et al.* 1978, Troiano *et al.* 1984,1987, Kesselring *et al.* 1989). Furthermore, Long and Holaday (1985) have demonstrated that CNS vascular permeability is increased in Adx rats and that this effect can be reversed by steroid replacement therapy, providing strong evidence that the integrity of the BBB is under the control of endogenous steroid hormones.

The location of lipocortin I in cells actively involved in the regulation of BBB function may indicate a role for this protein in control of BBB permeability, in MS and EAE, possibly through the influence of endogenous corticosteroids. Support for this suggestion is provided by the studies of Cirino *et al.* (1989) which have demonstrated that local administration of rh lipocortin I can mimic the prevention

of peripheral oedema by steroids in the rat. Furthermore, in a model of cerebral ischaemia Relton *et al.* (1991) showed that central injection of an N-terminal fragment of lipocortin I caused a marked inhibition of cerebral oedema, whereas administration of a neutralising antibody increased fluid accumulation, suggesting that lipocortin I may be an endogenous inhibitor of oedema in the CNS.

In summary, in the present study increased lipocortin I immunoreactivity was detected in CNS tissues from MS patients and EAE-diseased rats and was found to be associated with reactive astrocytes, and infiltrating lymphocytes and macrophages, respectively. In the case of EAE, an apparent relationship between the lipocortin I content of the CNS and serum corticosterone levels was observed, however this does not necessarily indicate steroid induction of the protein, since these findings could equally be due to an influx of lipocortin rich cells from the blood. Likewise, increased expression of the protein in reactive astroglia in MS may be elicited by glucocorticoids or may result from a glucocorticoid independent mechanism. In examining the role of lipocortins in steroid action, the next approach was to determine the effect of steroids on lipocortin levels in the CNS. It would be very interesting to study the influence of steroid therapy on lipocortin levels in MS brain tissue. Unfortunately such a study would be very difficult to perform because although corticosteroids are frequently used to treat acute attacks of relapsing MS, it is very uncommon for patients to be receiving steroids at the time of death. Hence post-mortem CNS tissue from steroid treated MS patients is not readily available. The animal model EAE however, is amenable to manipulation of steroid levels and thus was used to investigate the influence of exogenous and endogenous steroids on lipocortins in inflammatory CNS disease.

4.4 INFLUENCE OF EXOGENOUS AND ENDOGENOUS STEROIDS ON LIPOCORTIN LEVELS IN THE CNS DURING EAE

Studies so far had suggested that the increased levels of immunoreactive lipocortin I detected in the CNS of EAE-diseased animals might be induced by the rise in endogenous corticosteroids which is associated with spontaneous recovery from the disease. Therefore experiments were performed to investigate whether the amount of lipocortin I in CNS tissues can be influenced by artificial manipulation of serum corticosteroid levels during EAE.

In the first set of experiments animals were dosed with corticosteroids to determine if it was possible, by raising steroid levels, to cause a further induction of the protein. EAE-inoculated rats were treated therapeutically at the height of disease rather than prophylactically, because although prophylactic dosing has been shown to be very effective in controlling disease expression, CNS lesions do not develop (Rosenthale *et al.* 1969, Levine & Strebel 1969, Greig *et al.* 1970) and thus this would not provide a suitable model for studying the effect of steroids on lipocortins in the CNS. In addition, a fairly short treatment schedule of two or three doses over a 24 hour period was used because one well known action of steroids is to reduce leukocyte accumulation at inflammatory sites, and a decrease in the number of lipocortin-containing cells within the CNS tissue might mask any induction of the protein.

Initially corticosterone was studied because this is the main circulatory glucocorticoid hormone in the rat and therefore it is this compound which is thought to be responsible for self-cure and possibly the increase in CNS lipocortin levels. When animals were dosed at the height of disease with either 10 or 50 mg/kg corticosterone, no effect on lipocortins I, II or V was detected. However, RIA of serum corticosterone, which would measure both natural and injected hormone, revealed that at the higher dose, serum corticosterone concentrations

were significantly elevated. This indicated that substantial amounts of the drug were passing into the circulation and therefore probably into the CNS, since studies have shown the that steroids pass easily from the blood into the brain due to their high lipid solubility (Pardridge & Mietus 1979). Furthermore in EAE, breakdown of the BBB and oedema frequently occur in the vicinity of the lesions (Oldendorf & Towner 1974, Traugott *et al.* 1982, Daniel *et al.* 1981) and this increase in vascular permeability may also permit passage of blood borne substances into the CNS tissue.

It was considered possible that induction of lipocortin I had not been observed in these experiments due to dosing animals at the height of disease. During this phase circulatory steroids and CNS lipocortin levels are already elevated and it may be that further induction of lipocortin could not be achieved because the cells were already maximally stimulated. In view of this, in another experiment animals were treated with corticosterone immediately after complete recovery from neurological symptoms. At this stage some inflammatory cells are still present in the CNS, but serum corticosterone levels have returned to near basal. Thus cells might be sub-maximally stimulated and susceptible to further induction of lipocortin by administration of exogenous steroids. However, when animals were dosed in the recovery phase with 10 mg/kg corticosterone no change in CNS lipocortins was detected. Although it should be noted that circulating corticosterone concentrations were not found to be statistically different in drug and vehicle treated groups, making it unlikely that cells in the CNS were exposed to significantly increased steroid levels.

When animals were dosed at the height of disease with corticosterone although there appeared to be a small clinical effect at the lowest dose studied, this was not borne out in a subsequent experiment using a higher dose and a greater number of animals. In experiments designed to investigate whether the effects of

steroids may be mediated via induction of lipocortins, it would be preferable to induce an obvious clinical effect and then determine if lipocortin levels rise concomitantly. The doses of corticosterone used in the present study were calculated from a knowledge of the dose of dexamethasone shown to be effective in suppressing EAE (Bolton & Flower 1989), and the reported relative anti-inflammatory potencies of corticosterone and dexamethasone in the rat carrageenan paw oedema model (Flower & Dale 1989). It is possible that with a higher dose or a longer treatment schedule an effect on symptoms may have been achieved. However, corticosterone is known to have very weak anti-inflammatory properties (Flower & Dale 1989). Furthermore, Komarek & Dietrich (1971) found that 30 mg/kg corticosterone was ineffective in inhibiting EAE and that the compound had an LD_{50} of 35 mg/kg in the rat.

Since it appeared that it might prove difficult to induce a therapeutic effect in EAE using corticosterone, the influence of the synthetic steroid dexamethasone was investigated. Dexamethasone has potent anti-inflammatory properties and several studies have demonstrated that this compound is able to inhibit, suppress and treat EAE (Levine & Strebel 1969, Komarek & Dietrich 1971, Levine & Sowinski 1980, Bolton & Flower 1989). Doses used in the present study have been shown to be effective in the rat model by Bolton and Flower (1989), although animals were treated over a longer period of time than in the present study. Despite the reported efficacy of dexamethasone, when rats were dosed at the height of disease three times with 0.5 or 1 mg/kg no effect on symptoms was observed. This is probably due to the short duration of treatment. Levine and Sowinski (1980) observed that when rats were treated therapeutically with dexamethasone, recovery was accelerated slightly when three doses were given but not when one or two doses were injected, however, these workers used 5 mg/kg and the drug was administered over a three day period.

Although treatment of EAE-inoculated animals with dexamethasone had no effect on the symptoms of the disease, measurement of serum corticosterone showed that levels of the natural hormone were below the limit of detection in all dexamethasone treated animals except one. Control of circulating corticosteroids is regulated by a complex series of events. Secretion of glucocorticoids from the adrenal cortex is stimulated by ACTH released from the anterior pituitary, which in turn is regulated by corticotrophin-releasing factor (CRF) derived from the hypothalamus. Glucocorticoids exert a negative feedback on their own release by inhibiting secretion of both ACTH and CRF. The finding that serum corticosterone was significantly suppressed in dexamethasone treated animals, indicates that the drug is participating in negative feedback at the level of the pituitary and/or hypothalamus and strongly suggests that the compound was entering the CNS. The one dexamethasone treated rat in which very high serum corticosterone levels were detected appeared to be close to death when sampled and it is possible that in a moribund state, serum corticosterone escapes homeostatic control. Despite evidence that dexamethasone was entering the CNS, no effect on immunoreactive lipocortins I, II or V was detected.

The results of experiments with corticosterone and dexamethasone suggest that lipocortin levels in the CNS during EAE cannot be increased by dosing with additional steroids although it cannot be ruled out that a higher dose or in particular, a longer dosing schedule would have revealed an effect. Furthermore, since it has been hypothesised that steroid actions in EAE may be mediated through induction of lipocortins it would have been preferable to induce a clear clinical response to determine whether increased lipocortin levels are a prerequisite for such an effect. However, if steroid effects are mediated by induction of lipocortin, it might be expected that increases in the amount of protein would occur prior to improvement of symptoms. Moreover, it should be noted that in studies

where induction of lipocortin by steroids has been detected *in vivo* this has usually been observed within a few hours of treatment (Blackwell *et al.* 1980, Wallner *et al.* 1986, Smillie *et al.* 1989b, Goulding *et al.* 1990, Solito *et al.* 1990, Peers *et al.* 1993).

In a subsequent study an alternative approach to investigating the effects of steroids on CNS lipocortin levels was pursued. Rather than endeavouring to raise steroid levels, an attempt was made to block the effect of endogenous steroids by dosing animals with the glucocorticoid antagonist RU 38486. RU 38486 (mifepristone), is a potent competitive progesterone and glucocorticoid receptor antagonist (Philibert *et al.* 1981, Chrousos *et al.* 1984), which has anti-glucocorticoid activity both *in vitro* and *in vivo* (Chobert *et al.* 1983, Gagne *et al.* 1985). This compound has been shown to antagonise the immunosuppressive effects of glucocorticoids in a number of *in vitro* tests (Emilie *et al.* 1984) and to reverse the anti-inflammatory actions of dexamethasone in several experimental models of inflammation in the rat including pleurisy (Peers *et al.* 1988, Lelievre *et al.* 1988), adjuvant arthritis, paw oedema and peritonitis (Hirschelmann *et al.* 1988ab).

EAE-inoculated rats were dosed twice daily with 20 mg/kg RU 38486 on days 10-14 post-inoculation inclusive. Thus dosing was started just prior to the disease-associated rise in serum corticosterone and therefore the clinical and biochemical effects of this should have been blocked. Clinical scores of RU 38486 and vehicle treated EAE-inoculated animals were not statistically different, however this was probably due to the small sample sizes. Whereas vehicle treated animals appeared to be following the typical course of disease, all RU 38486 dosed rats were completely paralysed and appeared to be terminally ill at the time of sampling. The moribund condition of RU 38486 dosed animals suggests that the

drug was effectively antagonising the rise in corticosterone which appears to mediate self-cure.

Serum corticosterone levels in RU 38486 and vehicle dosed groups were not significantly different but there was a trend towards higher levels in rats treated with the antagonist, which again was probably not significant due to the low sample numbers. Other workers have observed that RU 38486 administration elevates circulating ACTH and cortisol in non-human primates and man due to blocking glucocorticoid negative feedback (Healy *et al.* 1983, Gaillard *et al.* 1984), and results suggest that this may have occurred to some extent in the present study.

If lipocortin I levels in the CNS are modulated by endogenous glucocorticoids, then it would be expected that a lower amount of the protein would be detected in RU 38486 dosed than vehicle treated groups. However, administration of the glucocorticoid did not appear to significantly influence lipocortin levels in the cervical spinal cord of either normal or EAE-inoculated animals. Although no effect on lipocortin was observed, it is not known if the drug was efficiently blocking glucocorticoid receptors in the CNS. However, studies have shown that when injected intravenously, radiolabelled RU 38486 accumulates in cell nuclei in steroid sensitive areas of the brain, suggesting that the compound readily penetrates the BBB (Coutard & Duval 1985). In addition, in a more extensive study in EAE using the same dosing regimen, Bolton and Flower (1989) found that RU 38486 significantly enhanced neurologic symptoms and reduced survival rate. Moreover they showed that the drug was able to reverse dexamethasone-induced suppression of the disease. Furthermore in the present study, the apparent effects of RU 38486 on clinical signs and serum corticosterone suggest that the compound was having a pharmacological effect and probably was causing some blockade of glucocorticoid receptors in the CNS.

Since there appeared to be no difference in the lipocortin content of cords from RU 38486 and vehicle treated animals this suggests that lipocortin levels in the CNS are not modulated by endogenous glucocorticoids. Moreover, since the amount of lipocortin I-like protein was elevated to the same extent in EAE-inoculated animals whether they were treated with the glucocorticoid antagonist or vehicle this suggests that endogenous corticosteroids are not responsible for mediating the increase in immunoreactive lipocortin I observed during EAE. However, because it was not possible to be sure that glucocorticoid receptors in the CNS were blocked in RU 38486 treated animals, the effect of adrenalectomy was investigated.

The adrenal cortex is the sole source of circulating corticosteroid hormones, thus Adx results in removal of all glucocorticoids from the blood. Assessment of serum corticosterone by RIA confirmed the success of adrenalectomy as values were below the limit of detection in most animals both at inoculation and at the time of sampling. When Adx rats were inoculated for EAE, they were found to be more susceptible to the disease than normal animals, succumbing several days early to a severe, rapidly progressive form of the disease. The increased susceptibility of Adx rats to EAE is well documented by other workers (Levine *et al.* 1962b, MacPhee *et al.* 1989), who report that death rapidly ensues unless animals are supplemented with glucocorticoids, indicating the critical importance of adrenal steroids in the mechanism of self-cure. Interestingly, at inoculation a small number of animals had low levels of circulating corticosterone, which was presumably due to incomplete removal of adrenal tissue. These animals did not succumb early to EAE, but instead went on to develop the disease at the same rate as non-operated controls. Thus it appears that this low level of corticosterone was able to delay onset until the normal time, again confirming the protective effect of endogenous corticosteroids.

In contrast, sham-operated rats appeared to be relatively resistant to the disease. In a preliminary experiment, sham-operated animals were found to develop a delayed, milder type of EAE than that which is normally observed in the Lewis rat. It is possible that this effect was also due to adrenal steroids. Various forms of stress have been shown to inhibit the development of EAE (Levine *et al.* 1962a, Bukilica *et al.* 1991). Although at least one week was allowed between surgery and inoculation, it may be that the physical trauma of the sham operation had a suppressive influence on the induction of the disease, possibly due to stimulating release of glucocorticoids. Measurement of serum corticosterone however appeared to yield conflicting results, on the one hand, at inoculation, levels in sham-operated animals were not significantly higher than in non-operated animals (although there may have been a marginal difference). On the other, levels in sham-operated animals decreased slightly (significantly in the third experiment), from inoculation to the day of sampling. This may indicate that circulating corticosterone was elevated above normal at inoculation, however, the different sampling techniques used at each time point should also be considered. At inoculation rats were anaesthetised with halothane and 1 ml of blood taken slowly by cardiac puncture. At the end of the experiments 2 ml of blood was collected relatively quickly, again by cardiac puncture, but from animals which were rendered unconscious by CO_2 inhalation. Various types of anaesthesia are known to stimulate release of corticosteroids to a greater or lesser extent (Hedner & Rerup 1960, Cook *et al.* 1973, S.H. Peers and R.J. Flower, personal communication) and it is possible that the apparent decrease in circulating corticosteroids in sham-operated animals between the time of inoculation and the time of sampling may be an artefact resulting from the influence of anaesthesia and/or the time taken to collect the sample.

Assessment of lipocortins in cervical spinal cord samples taken from Adx, sham-operated and non-operated rats, revealed no difference in the levels of immunoreactive lipocortins II and V, but marked differences in lipocortin I immunoreactivity. In both experiments the amount of lipocortin I-like protein was considerably elevated in Adx rats compared to sham-operated and non-operated controls. In addition, in one experiment levels of immunoreactive lipocortin I in cords from sham-operated animals appeared to be lower than in non-operated rats, although in the other experiment this difference was only marginal.

The detection of lower levels of lipocortin I-like protein in CNS tissues from sham-operated animals is perhaps not surprising. In previous experiments it was observed that the amount of immunoreactive lipocortin I in the CNS during EAE appears to be closely related to both the stage of disease and the number of lesions. Sham-operated rats were sampled on day 10 PI, these animals appeared to be relatively resistant to the induction of EAE, but even in the normal course of events, only a few small, early lesions would be present in the CNS at this stage. In contrast, both Adx and non-operated rats were sampled at the height of disease and would therefore have developed extensive lesions in the CNS. Thus differences between sham-operated and other groups are probably a reflection of the number of infiltrating lipocortin-containing cells in the CNS.

The higher levels of lipocortin I immunoreactivity observed in CNS tissues from Adx rats inoculated for EAE were completely unexpected. These animals had no (or virtually no) circulating corticosteroids both at the time of inoculation and at the end of the experiment. If the elevation in immunoreactive lipocortin I seen in the CNS of EAE inoculated rats is due to induction by endogenous glucocorticoids, then Adx rats would be expected to exhibit lower levels of the protein than non-operated controls. Interestingly, elevated levels of lipocortins have been detected in Adx animals by other workers. In an early study, Flower

(1984) found increased macrocortin in peritoneal lavage cells from Adx rats than in sham-operated controls, which was attributed to a diminished rate of secretion of the protein secondary to the lack of endogenous steroids. In addition, in another study on the effect of Adx on lipocortins in peritoneal leukocytes Peers *et al.* (1993) also reported encountering instances where the amount of lipocortin I present in cells from Adx rats was high and suggested that the trauma of surgery or a sub-clinical infection following the operation might activate cells to synthesise more lipocortin I through a non-glucocorticoid control mechanism.

Whilst it is possible that in the present study increased levels of lipocortin I-like protein may be due to accumulation of the protein caused by lack of secretion, or that surgical trauma and associated release of humoural factors may have activated cells to produce lipocortins, there are other possible explanations for the results observed. Adx rats are known to exhibit an exaggerated inflammatory response to irritants compared to sham-operated animals. Fluid exudation, leukocyte infiltration and release of eicosanoids in response to carrageenin have been demonstrated to be greatly enhanced in the absence of endogenous steroids (Parente & Flower 1985b, Flower *et al.* 1986). Furthermore, endogenous corticosteroids also appear to be important for maintaining the patency of the BBB (Long & Holaday 1985). Since Adx rats were found to develop a more severe form of EAE, it may be envisaged that this was due to lack of endogenous steroids resulting in an enhanced inflammatory response in the CNS and that this was accompanied by passage of larger numbers of cells across the BBB. Thus the increased levels of lipocortin I in the CNS of EAE-diseased rats may simply be due to an increased number of infiltrating cells.

An alternative explanation arises from the effects that steroids are known to have on the distribution of circulating leukocytes, between the blood, lymphoid organs and other tissues. Administration of glucocorticoids causes

lymphocytopenia, monocytopenia and neutrophilia (Fauci 1979). Presumably removal of endogenous corticosteroids may also result in some redistribution of these cell types. Since circulating leukocytes have been shown to contain significant amounts of lipocortin I (Comera *et al.* 1989,1990, Goulding *et al.* 1990) it is conceivable that adrenalectomy may cause a change in the levels of lipocortin I in the blood by altering the number or relative proportions of leukocytes and that this might be detected as an apparent increase in the CNS tissue.

In summary, if steroids do exert their anti-inflammatory effects in EAE via induction of lipocortins in the CNS, then it would be expected that steroid treatment of EAE-diseased animals might cause a further increase in lipocortins and that treatment with RU 38486 or adrenalectomy would cause a decrease in the amount of lipocortins detected in CNS tissues from these animals. No change in lipocortins was detected in the CNS of EAE-diseased rats following treatment with corticosterone or dexamethasone, suggesting that lipocortins cannot be upregulated by exogenous glucocorticoids. It is unfortunate that in these studies no effect on symptoms was observed because this would have confirmed whether enhanced expression of lipocortin was a pre-requisite for clinical effect. However measurement of serum corticosterone suggested that with respect to corticosterone the drug was passing into the circulation, and in the case of dexamethasone, that it was also probably entering the CNS, since suppression of the HPA axis was observed. In a study using the glucocorticoid antagonist RU 38486 no significant alteration in lipocortin levels was found, suggesting that endogenous glucocorticoids do not modulate lipocortins in the CNS in either normal or EAE-diseased animals. However it was not proven that glucocorticoid receptors in the CNS were blocked. The results of the Adx experiment are particularly interesting since a clear clinical effect was observed and there can be no doubt that steroid levels were significantly altered. Since lipocortin levels were not down regulated in

Adx animals compared to controls this provides strong evidence that the amount of lipocortin in the CNS is not modulated by glucocorticoids. Furthermore, that the increase in lipocortin I in the CNS of EAE-diseased rats although occurring at the same time as, is not caused by, changes in serum corticosterone.

The steroid inducibility of lipocortins is a highly controversial subject (see introduction section: 1.3.3.2). The large number of studies which have investigated this problem have frequently produced conflicting results. There have been few reports on the effects of steroids on lipocortins in the CNS, but those which exist are not at variance with results observed in the present study. Using Western blotting, Smith *et al.* (1989b) detected no effect of Adx or dexamethasone treatment on the levels of lipocortins I and V either in steroid sensitive components of the HPA axis (adenohypophysis, hypothalamus and hippocampus) or in control areas (striatum and cortex) in the rat. Furthermore in a subsequent study by ELISA they observed no influence of the steroid on lipocortin I in specific brain nuclei (Smith *et al.* 1990c). Using immunohistochemistry to localise immunoreactive lipocortin I in the rat CNS, Strijbos and co-workers (1991) found that 2 hr peripheral administration of dexamethasone had no effect on the distribution of the protein in any areas or cell-types in the brain, but noted that Adx reduced neuronal staining in the hippocampus although no effect on any other brain region was detected.

Since in immunohistochemistry experiments it was found that lipocortin I immunoreactivity in the CNS of EAE-diseased rats is located predominantly in infiltrating lymphocytes and macrophages, studies on these cell-types are also relevant. Several workers have observed induction of lipocortins I (Wallner *et al.* 1986), I and II (Smillie *et al.* 1989b, Peers *et al.* 1993), and I, II and V (Solito *et al.* 1990), in rat peritoneal leukocytes following *in vivo* treatment with glucocorticoids. Furthermore it has been demonstrated that dosing animals with

RU 38486 reduces lipocortins I and II (Smillie *et al.* 1989b) or reverses steroid induction of lipocortins I and II in these cells (Peers *et al.* 1993). Induction of lipocortin I in human peripheral blood mononuclear cells following administration of glucocorticoids has also been reported (Goulding *et al.* 1990). However there have been a significant number of studies where the authors have been unable to find induction of lipocortins by glucocorticoids in macrophages and lymphocytes *in vitro* (Northup *et al.* 1988, Bronnegard *et al.* 1988) and *in vivo* (Wong *et al.* 1991). Thus although there are no studies which are directly applicable, there are reports which both support and contradict results observed in the present study.

It should be noted that although no change was detected in the absolute amount of immunoreactive lipocortins I, II and V in CNS tissues following manipulation of serum corticosteroid levels, it cannot be ruled out that the treatments studied may have had an effect which was not detectable by the methods used. Western blotting is not sensitive to small changes in protein levels and probably would not pick up small variations in lipocortin, or a large alteration occurring in a small number of cells, although obviously these may be physiologically important. It would therefore be interesting to investigate whether such changes might be detected using immunohistochemistry to visualise lipocortins in different cell-types. Furthermore, it has recently been suggested that steroids may alter the subcellular localisation of lipocortins and that steroid treatment may cause increased expression of lipocortin on the cell surface where it may be biologically active (Smillie *et al.* 1989b, Goulding *et al.* 1990, Browning *et al.* 1990, Peers *et al.* 1993). Again this would not be detectable using Western blotting, but could be investigated using appropriate immunostaining and electron microscopical techniques.

4.5 CONCLUSIONS

Anti-inflammatory steroids are frequently used to treat acute relapse of MS because of their ability to hasten recovery from debilitating symptoms. These compounds are also effective in suppressing the animal counterpart, EAE. Furthermore, it appears that endogenous corticosteroids are responsible for mediating spontaneous recovery from EAE in the rat and there is some evidence that circulatory steroids may influence disease activity in MS. The mechanism though which steroids exert their effects in MS and EAE is unknown but steroids possess anti-inflammatory, anti-oedematous and immunomodulatory properties which potentially may all play a role in their actions in these diseases. Recently it has been proposed that the anti-inflammatory effects of corticosteroids may be mediated in part by the induction of "second messenger" proteins termed lipocortins. The present study has investigated whether the potential exists for lipocortins to play a role in the mechanism of action of steroids in MS and EAE.

The first aim of this project was to investigate the occurrence of lipocortins in the CNS of patients with MS and rats during EAE. Initial experiments indicated that CSF was not suitable for the study of lipocortins in these diseases. However, investigation of lipocortins in CNS tissue samples by Western blotting revealed the presence of immunoreactive lipocortins I, II, IV and V in normal human CNS tissues and lipocortins I, II and V in rat CNS samples. Furthermore, increased levels of all lipocortins were detected in diseased CNS tissues from MS patients and an elevated amount of immunoreactive lipocortin I in the CNS of rats with EAE. These observations indicate that the potential exists for lipocortins to be involved in the modulation of MS and EAE.

The second aim was to shed light on the functions of lipocortins in the CNS by studying the cellular localisation of these proteins. Using immunohistochemical

techniques, lipocortin I immunoreactivity was observed to be located primarily in the walls of blood vessels in normal human and rat CNS tissues. The increased levels of lipocortin I-like protein detected in the CNS in MS and EAE by Western blotting, were found to be due to expression of the protein by reactive astrocytes, and widespread immunostaining of infiltrating lymphocytes and macrophages, respectively. All the cell-types in which immunoreactive lipocortin I was detected are known to be of central importance in the pathology of these diseases. Lymphocytes and macrophages are key effector cells of the immune response and phagocytic cells are also believed to mediate demyelination. Endothelial cells are a major component of the BBB which under normal circumstances maintains exclusion of circulating immune elements but in MS and EAE becomes permeable allowing passage of immunocompetent cells and humoral mediators into the CNS. Astrocytes too, form part of the BBB and also appear to modulate immune reactivity within the CNS and to mediate tissue repair following damage. Thus the observation of often high levels of expression of lipocortin I immunoreactivity within these cells suggests that the protein may play a role in the functioning of cells which are central to the pathology of MS and EAE. In these experiments, corticosterone levels in the serum of EAE-inoculated rats was also determined and it was found that increases in immunoreactive lipocortin I in the CNS coincided with changes in circulatory corticosterone, suggesting that endogenous corticosteroids may be causing induction of lipocortin I in the CNS during EAE.

The third aim of the present study was to explore the involvement of lipocortins in the mechanism of steroid action by investigating whether manipulation of corticosteroids in EAE-inoculated animals would induce a change in CNS lipocortin levels. Thus, circulating glucocorticoids were raised by administration of corticosterone or dexamethasone, antagonised by treatment with RU 38486, or abolished by Adx prior to inoculation. However corticosterone and

dexamethasone were found to have no effect on lipocortin levels in the cervical spinal cord and RU 38486 and Adx did not prevent the accumulation of lipocortin I-like protein observed during the course of the disease. These results suggest that steroids do not modulate the absolute amount of lipocortins in CNS tissue, and furthermore, that the increase in immunoreactive lipocortin I found in the cervical spinal cord of EAE-diseased rats is not due to induction of the protein by endogenous steroids. However it is not proven that steroids had no effect on lipocortins as it cannot be ruled out that steroids caused a more subtle alteration in lipocortins which could not be detected using the techniques in this study. Moreover, neither do these findings prove that steroid effects are not mediated by lipocortins.

What is the function of lipocortins in the CNS in MS and EAE? The lipocortin theory states that lipocortins are steroid-inducible proteins which inhibit the activity of PLA_2, thus preventing generation of active arachidonic acid metabolites and thereby mediating the anti-inflammatory effects of steroids. Whilst it is possible that lipocortins may play such a role in MS and EAE, many other functions have been postulated for various members of the annexin family (see introduction section: 1.3.3) and the function of any one of these proteins has yet to be determined. Recently some very interesting work has emerged which is particularly relevant to the present study, demonstrating that lipocortin I has biological activity in the CNS. Intravenously administered rh lipocortin I has been shown to mimic the anti-pyretic effect of dexamethasone by preventing the febrile response to poly I:C in the rabbit (Davidson *et al.* 1991). Furthermore, in studies in the rat Carey *et al.* (1990) have demonstrated that not only does central injection of a recombinant fragment (N-terminal 1-188) of lipocortin I reproduce the effect of dexamethasone in inhibiting cytokine-induced fever and thermogenesis, but also that the suppressive action of the steroid on the pyrogenic response can be

reversed by administration of a neutralising antibody to the peptide, findings which suggest that lipocortin I may be a physiological mediator of the anti-pyretic actions of corticosteroids. Evidence has also accumulated that endogenous lipocortin I may be an inhibitor of oedema and protect against neuronal damage in the CNS. In a rodent model of cerebral ischaemia induced by occlusion of the middle cerebral artery, Relton *et al.* (1991) have observed increased expression of immunoreactive lipocortin I around infarcted areas. In addition, these workers found that central injection of the 1-188 N-terminal fragment of lipocortin I reduced infarct size and the extent of oedema, whereas administration of neutralising antibody significantly potentiated ischaemic damage and oedema. Lipocortin I fragment 1-188 has also been shown to attenuate lesions in the rat CNS caused by NMDA receptor agonists and again in this model the neutralising antibody was found to increase neuronal damage (Black *et al.* 1992). Taken together these studies strongly suggest a physiological role for lipocortins in the central actions of glucocorticoids on pyrogenesis and as an endogenous inhibitor of tissue damage in the brain.

Understanding the role played by lipocortins in the action of steroids in MS and other inflammatory diseases is of importance as it may lead to the development of new therapies which are free of the side effects which so frequently limit the use of these potent compounds. Findings in the present study suggest that steroids do not modulate the absolute amounts of lipocortins I, II and V in the CNS in EAE, which must raise a question mark over whether lipocortins do mediate the beneficial effects of glucocorticoids in inflammatory CNS disease. However, the presence of these proteins in cell-types central to the pathology of MS and EAE may indicate a role for lipocortins in these diseases. Further work is required to determine whether steroid effects are mediated by a more subtle modulation of lipocortins and to elucidate the function of these fascinating but elusive proteins.

REFERENCES

Aarsman, A.J., Mynbeek, G., van den Bosch, H., Rothhut, B., Prieur, B., Comera, C., Jordan, L. and Russo-Marie, F. (1987) Lipocortin inhibition of extracellular and intracellular phospholipases A_2 is substrate concentration dependent. FEBS Lett. 219, 176-180.

Abbruzzese, G., Gandolfo, C. and Loeb, C. (1983) 'Bolus' methylprednisolone versus ACTH in the treatment of multiple sclerosis. Ital.J.Neurol.Sci. 4, 169-172.

Adams, C.W.M. (1976) The progression of the lesion in multiple sclerosis. Neurology 26, 33-34.

Adams, C.W.M. (1977) Pathology of multiple sclerosis: progression of the lesion. Br.Med.Bull. 33, 15-20.

Adams, C.W.M., Poston, R.N. and Buk, S.J. (1989) Pathology, histochemistry and immunocytochemistry of lesions of acute multiple sclerosis. J.Neurol.Sci. 92, 291-306.

Adams, J.M. and Imagawa, D.T. (1962) Measles antibodies in multiple sclerosis. Proc.Soc.Exp.Biol.Med. 111, 562-566.

Ahn, N.G., Teller, D.C., Bienkowski, M.J., McMullen, B.A., Lipkin, E.W. and de Haen, C. (1988) Sedimentation equilibrium analysis of five lipocortin-related phospholipase A_2 inhibitors from human placenta. J.Biol.Chem. 263, 18657-18663.

Alexander, L., Cass, L.J. and Zuniga, J.A. (1971) ACTH-induced adrenocortical response patterns in multiple sclerosis and their relation to the effectiveness of ACTH therapy. Confin.Neurol. 33, 1-24.

Ali, S.M., Geisow, M.J. and Burgoyne, R.D. (1989) A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. Nature 340, 313-315.

Allen, I.V. and McKeown, S.R. (1979) A histological, histochemical and biochemical study of the macroscopically normal white matter in multiple sclerosis. J.Neurol.Sci. 41, 81-91.

Allen, J., Powers, C., Kepic, T., Talareco, J., Garwacki, D. and Swank, R. (1980) Plasma peptide concentrations in patients with multiple sclerosis. Clin.Res. 28, 255A.

Alter, M., Kahana, E. and Loewenson, R. (1978) Migration and risk of multiple sclerosis. Neurology 28, 1089-1093.

Ambrose, M.P. and Hunninghake, G.W. (1990a) Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. J.Appl.Physiol. 68, 1668-1671.

Ambrose, M.P. and Hunninghake, G.W. (1990b) Corticosteroids increase lipocortin I in alveolar epithelial cells. Am.J.Respir.Cell Mol.Biol. 3, 349-353.

Ando, Y., Imamura, S., Hong, Y-M., Owada, M.K., Kakunaga, T. and Kannag, R. (1989) Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analysed by phospholipid affinity column chromatography. J.Biol.Chem. 264, 6948-6955.

Ando, Y., Imamura, S., Owada, M.K. and Kannagi, R. (1991) Calcium-induced intracellular cross-linking of lipocortin I by tissue transglutaminase in A431 cells. J.Biol.Chem. 266, 1101-1108.

Armelin, M.C.S. and Armelin, H.A. (1983) Glucocorticoid hormone modulation of both cell surface and cytoskeleton related to growth control of rat glioma cells. J.Cell Biol. 97, 459-465.

Arnason, B.G., Jankovic, B.D., Waksman, B.H. and Wennersten, C. (1962) Role of the thymus in immune reactions in rats: II. Suppressive effect of thymectomy at birth on reactions of delayed (cellular) hypersensitivity and the circulating small lymphocyte. J.Exp.Med. 116, 117-186.

Arnason, B.G.W. and Chelmicka-Schorr, E. (1974) Peripheral nerve sequential demyelination induced by intraneural diphtheria toxin injection. 1. Effect of hydrocortisone as measured by muscle twitch tension. Arch.Neurol. 30, 157-162.

Arnason, S.M., Bauer, H.J. and Brown, J.R. (1982) Therapeutic claims in multiple sclerosis, International Federation of Multiple Sclerosis Societies, New York, pp. 1-297.

AUSTIMS, (1989) Interferon-α and transfer factor in the treatment of multiple sclerosis: a double-blind, placebo-controlled trial. J.Neurol.Neurosurg.Psychiatry 52, 566-574.

Bansil, S., Troiano, R., Cook, S.D. and Rohowsky-Kochan, C. (1991) Serum soluable interleukin-2 receptor levels in chronic progressive, stable and steroid-treated multiple sclerosis. Acta Neurol.Scand. 84, 282-285.

Barkhof, F., Hommes, O.R., Scheltens, P. and Valk, J. (1991) Quantitative MRI changes in gadolinium-DTPA enhancement after high-dose intravenous methylprednisolone in multiple sclerosis. Neurology 41, 1219-1222.

Barnes, M.P., Bateman, D.E., Cleland, P.G., Dick, D.J., Walls, T.J., Newman, P.K., Saunders, M. and Tilley, P.J.B. (1985) Intravenous methylprednisolone for multiple sclerosis in relapse. J.Neurol.Neurosurg.Psychiatry 48, 157-159.

Bastian, B.C., Van der Piepen, U.A., Romisch, J., Paques, E. and Burg, G. (1991) Immunohistochemical localization of proteins of the lipocortin/annexin family in normal and diseased human skin. J.Invest.Dermatol. 96, P38.

Batchelor, J.R., Compston, A. and McDonald, W.I. (1978) The significance of the association between HLA and multiple sclerosis. Br.Med.Bull. 34, 279-284.

Baxter, J.D. (1976) Glucocorticoid hormone action. In: Gill, G.N. (ed.) Pharmacology and therapeutics. Part B, Pergamon Press, Oxford, pp. 605-659.

Ben-Nun, A., Wekerle, H. and Cohen, I.R. (1981) Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. Nature 293, 60-61.

Besedovsky, H., Sorkin, E., Keller, M. and Muller, J. (1975) Changes in blood hormone levels during the immune response. Proc.Soc.Exp.Biol.Med. 150, 466-470.

Beyaert, R., Suffys, P., Van Roy, F. and Fiers, W. (1990) Inhibition by glucocorticoids of tumor necrosis factor-mediated cytotoxicity. Evidence against lipocortin involvement. FEBS Lett. 262, 93-96.

Bianchi, R., Giambanco, I., Ceccarelli, P., Pula, G. and Donato, R. (1992) Membrane-bound annexin V isoforms (CaBP33 and CaBP37) and annexin VI in bovine tissues behave like integral membrane proteins. FEBS Lett. 296, 158-162.

Bienkowski, M.J., Petro, M.A. and Robinson, L.J. (1989) Inhibition of thromboxane A synthesis in U937 cells by glucocorticoids. Lack of evidence for lipocortin 1 as the second messenger. J.Biol.Chem. 264, 6536-6544.

Black, M.D., Carey, F., Crossman, A.R., Relton, J.K. and Rothwell, N.J. (1992) Lipocortin-1 inhibits NMDA receptor-mediated neuronal damage in the striatum of the rat. Brain Res. 585, 135-140.

Blackwell, G.J., Flower, R.J., Nijkamp, F.P. and Vane, J.R. (1978) Phospholipase A_2 activity of guinea-pig isolated perfused lungs: stimulation and inhibition by anti-inflammatory steroids. Br.J.Pharmacol. 62, 79-89.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J., Parente, L. and Persico, P. (1980) Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. Nature 287, 147-149.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J., Langham, C.S.J., Parente, L., Persico, P., Russell-Smith, N.C. and Stone, D. (1982) Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. Br.J.Pharmacol. 76, 185-194.

Blackwell, G.J. (1983) Specificity and inhibition of glucocorticoid-induced macrocortin secretion from rat peritoneal macrophages. Br.J.Pharmacol. 79, 587-594.

Blaw, M., Cooper, M.D. and Good, R.A. (1967) Experimental allergic encephalomyelitis in agammaglobinemic chickens. Science 158, 1198-1200.

Bohn, H., Kraus, W. and Winkler, W. (1985) Isolation and characterization of 4 new placental tissue proteins (pp18, pp19, pp20, pp21). Arch.Gynaecol. 236, 225-233.

Bolton, C. and Cuzner, M.L. (1980) Modification of experimental allergic encephalomyelitis by non-steroidal anti-inflammatory drugs. In: Davison, A.N. and Cuzner, M.L. (eds.) The suppression of experimental allergic encephalomyelitis and multiple sclerosis, London, pp. 189-197.

Bolton, C., Allsopp, G. and Cuzner, M.L. (1982a) The effect of cyclosporin A on the adoptive transfer of experimental allergic encephalomyelitis in the Lewis rat. Clin.Exp.Immunol. 47, 127-132.

Bolton, C., Borel, J.F., Cuzner, M.L., Davison, A.N. and Turner, A.M. (1982b) Immunosuppression by cyclosporin A of experimental allergic encephalomyelitis. J.Neurol.Sci. 56, 147-153.

Bolton, C., Gordon, D. and Turk, J.L. (1984a) Prostaglandin and thromboxane levels in central nervous system tissues from rats during the induction and development of experimental allergic encephalomyelitis (EAE). Immunopharmacology 7, 101-107.

Bolton, C., Turner, A.M. and Turk, J.L. (1984b) Prostaglandin levels in cerebrospinal fluid from multiple sclerosis patients in remission and relapse. J.Neuroimmunol. 6, 151-159.

Bolton, C. and Flower, R.J. (1989) The effects of the anti-glucocorticoid RU 38486 on steroid-mediated suppression of experimental allergic encephalomyelitis (EAE) in the Lewis rat. Life Sci. 45, 97-104.

Bolton, C., Elderfield, A.-J. and Flower, R.J. (1990) The detection of lipocortins 1, 2 and 5 in central nervous system tissues from Lewis rats with acute experimental allergic encephalomyelitis. J.Neuroimmunol. 29, 173-181.

Bolton, C. (1992) The efficacy of cyclosporin A, FK-506 and prednisolone to modify the adoptive transfer of experimental allergic encephalomyelitis (EAE). Agents Actions 35, 79-84.

Borel, J.F., Feurer, C., Gubler, H.U. and Stahelin, H. (1976) Biological effects of cyclosporin A: a new anti-lymphocytic agent. Agents Actions 6, 468-475.

Borgeson, M., Tallent, M.W. and Keane, R. (1989) Astrocyte modulation of central nervous system immune responses. In: Neuroimmune networks: Physiology and diseases, Alan R.Liss, New York, pp. 51-55.

Bornstein, M.B., Miller, A., Slagle, S., Weitzman, M., Crystal, H., Drexler, E., Keilson, M., Merriam, A., Wassertheilsmoller, S., Spada, V., Weiss, W., Arnon, R., Jacobsohn, I., Teitelbaum, D. and Sela, M. (1987) A pilot trial of COP 1 in exacerbating-remitting multiple sclerosis. New.Engl.J.Med. 317, 408-414.

Bostock, H. and McDonald, W.I. (1982) Recovery and function after demyelination. In: Sears, T.A. (ed.) Neuronal-glial cell interrelationships, Dahlen Konferenzen, Springer Verlag, New York, pp. 287-301.

Boustead, C.M., Walker, J.H., Kennedy, D. and Waller, D.A. (1991) Crystallization and preliminary X-ray studies of annexin IV (endonexin), a calcium-dependent phospholipid-binding protein. FEBS Lett. 279, 187-189.

Bowen, D.M. and Davison, A.N. (1974) Macrophages and cathepsin-A activity in multiple sclerosis brain. J.Neurol.Sci. 21, 227-231.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. 72, 248-254.

Brambilla, F., Cazzullio, C.L., Smeraldi, E. and Zibetti, A. (1974) Endocrine function in multiple sclerosis: possible correlation with immunitary phenomena. Acta Neurol.(Napoli) 29, 618-625.

Brenner, T., Boneh, A., Shohami, E., Abramsky, O. and Weidenfeld, J. (1992) Glucocorticoid regulation of eicosanoid production by glial cells under basal and stimulated conditions. J.Neuroimmunol. 40, 273-280.

British and Dutch Multiple Sclerosis Azathioprine Trial Group (1988) Double-masked trial of azathioprine in multiple sclerosis. Lancet 2, 179-183.

Bronnegard, M., Andersson, O., Edwall, D., Lund, J., Norstedt, G. and Carlstedt-Duke, J. (1988) Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol.Endocrinol. 2, 732-739.

Browning, J.L., Ward, M.P., Wallner, B.P. and Pepinsky, R.B. (1990) Studies on the structural properties of lipocortin-1 and the regulation of its synthesis by steroids. In: Melli, M. and Parente, L. (eds.) Cytokines and lipocortins in inflammation and differentiation, Wiley-Liss, New York, pp. 27-45.

Buckley, C., Kennard, C. and Swash, M. (1982) Treatment of acute exacerbations of multiple sclerosis with intravenous methylprednisolone. J.Neurol.Neurosurg.Psychiatry 45, 179-180.

Bukilica, M., Djordjevic, S., Maric, I., Dimitrijevic, M., Markovic, B.M. and Jankovic, B.D. (1991) Stress-induced suppression of experimental allergic encephalomyelitis in the rat. Intern.J.Neuroscience 59, 167-175.

Buller, R.E. and O'Malley, B.W. (1976) The biology and mechanism of steroid hormone receptor interaction with the eukaryotic nucleus. Biochem.Pharmacol. 25, 1-12.

Burgoyne, R.D., Cambray-Deakin, M.A. and Norman, K-M. (1989) Developmental regulation of tyrosine kinase substrate p36 (calpactin heavy chain) in rat cerebellum. J.Mol.Neurosci. 1, 47-54.

Burnham, J.A., Wright, R.R., Dreisbach, J. and Murray, R.S. (1991) The effect of high-dose steroids on MRI gadolinium enhancement in acute demyelinating lesions. Neurology 41, 1349-1354.

Burns, A.L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M.R. and Pollard, H.B. (1989) Calcium channel activity of purified human synexin and structure of the human synexin gene. Proc.Natl.Acad.Sci.USA 86, 3798-3802.

Calignano, A., Carnuccio, R., Di Rosa, M., Ialenti, A. and Moncada, S. (1985) The anti-inflammatory effect of glucocorticoid-induced phospholipase inhibitory proteins. Agents Actions 16, 60-62.

Cammer, W., Bloom, B.R., Norton, W.T. and Gordon, S. (1978) Degradation of basic protein in myelin by neutral proteases secreted by stimulated macrophages: A possible mechanism of inflammatory demyelination. Proc.Natl.Acad.Sci.USA 75, 1554-1558.

Cammer, W., Tansey, F.A. and Brosnan, C.F. (1990) Reactive gliosis in the brains of Lewis rats with experimental allergic encephalomyelitis. J.Neuroimmunol. 27, 111-120.

Campbell, B., Vogel, P.J., Fischer, E. and Lorenz, R. (1973) Myelin basic protein administration in multiple sclerosis: Arch.Neurol. 29, 10-15.

Carey, F., Forder, R., Edge, M.D., Greene, A.R., Horan, M.A., Strijbos, P.J.L.M. and Rothwell, N.J. (1990) Lipocortin 1 fragment modifies pyrogenic actions of cytokines in rats. Am.J.Physiol. 259, R266-R269.

Carnuccio, R., Di Rosa, M. and Persico, P. (1980) Hydrocortisone induced inhibitor of prostaglandin biosynthesis in rat leucocytes. Br.J.Pharmacol. 68, 14-16.

Carnuccio, R., Di Rosa, M., Flower, R.J. and Pinto, A. (1981) The inhibition by hydrocortisone of prostaglandin biosynthesis in rat peritoneal leucocytes is correlated with intracellular macrocortin levels. Br.J.Pharmacol. 74, 322-324.

Charcot, J.M. (1877) Lectures on the diseases of the nervous system, New Sydenham Society, London.

Chauh, S.Y. and Pallen, C.J. (1989) Calcium-dependent and phosphorylation-stimulated proteolysis of lipocortin 1 by an endogenous A431 cell membrane protease. J.Biol.Chem. 264, 21160-21166.

Chobert, M-N., Barouki, R., Finidori, J., Aggerbeck, M., Hanoune, J., Philibert, D. and Deraedt, R. (1983) Antiglucocorticoid properties of RU 38486 in a differentiated hepatoma cell line. Biochem.Pharmacol. 32, 3481-3483.

Christmas, P., Callaway, J., Fallon, J., Jones, J. and Haigler, H.T. (1991) Selective secretion of annexin I, a protein without a signal sequence, by the human prostate gland. J.Biol.Chem. 266, 2499-2507.

Chrousos, G.P., Nieman, L., Healy, D., Spitz, I., Hodgen, G., Bardin, C.W., Cutler, G.B., Schulte, H.M., Merriam, G.R., Brandon, D.D. and Loriaux, D.L. (1984) Antiglucocorticoids: general aspects and clinical implications. In: Fehm, H.L., Graupe, K. and Kobberling, J. (eds.) Glukokortikoide: Forschung und therapie, Fachbuch-Verlagsgesellschaft, Erlangen, pp. 54-71.

Chung, I.Y. and Benveniste, E.N. (1990) Tumor necrosis factor-alpha production by astrocytes: induction by lipopolysaccharide, interferon-gamma and interleukin-1. J.Immunol. 144, 2999-3007.

Cirino, G. and Flower, R.J. (1987a) Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery *in vitro*. Prostaglandins 34, 59-62.

Cirino, G. and Flower, R.J. (1987b) The inhibitory effect of lipocortin on eicosanoid synthesis is dependent on Ca^{2+} ions. Br.J.Pharmacol. 92, 521P.

Cirino, G., Flower, R.J., Browning, J.L., Sinclair, L.K. and Pepinsky, R.B. (1987) Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. Nature 328, 270-272.

Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L. and Pepinsky, R.B. (1989) Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. Proc.Natl.Acad.Sci.USA 86, 3428-3432.

Cloix, J.F., Colard, O., Rothhut, B. and Russo-Marie, F. (1983) Characterization and partial purification of 'renocortins': two polypeptides formed in renal cells causing the anti-phospholipase-like action of glucocorticoids. Br.J.Pharmacol. 79, 313-321.

Coggi, G., Dell'Orto, P. and Viale, G. (1986) Avidin-biotin methods. In: Polak, J.M. and Van Noorden, S. (eds.) Immunocytochemistry. Modern methods and applications, John Wright and Sons, Bristol, Ed. 2nd, pp. 54-70.

Comera, C., Rothhut, B., Cavadore, J.C., Vilgrain, I., Cochet, C., Chambaz, E. and Russo-Marie, F. (1989) Further characterization of four lipocortins from human peripheral blood mononuclear cells. J.Cell.Biochem. 40, 316-370.

Comera, C., Rothhut, B. and Russo-Marie, F. (1990) Identification and characterization of phospholipase A_2 inhibitory proteins in human mononuclear cells. Eur.J.Biochem. 188, 139-146.

Compston, D.A.S., Milligan, N.M., Hughes, P.J., Gibbs, J., McBroom, V., Morgan, B.P. and Campbell, A.K. (1987) A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis: 2. laboratory results. J.Neurol.Neurosurg.Psychiatry 50, 517-522.

Condie, R.M. and Good, R.A. (1959) Experimental allergic encephalomyelitis: Its production, prevention and pathology as studied by light and electron microscopy. In: Korey, S.R. (ed.) The biology of myelin, Harper(Hoeber), New York, pp. 321-384.

Confavreux, C., Thivolet, C., Ventre, J.J., Aimard, G. and Devic, M. (1986) Treatment of multiple sclerosis with isoprinosine - 52 cases. Presse Med. 15, 2256-2257.

Cook, D.M., Kendall, J.W., Greer, M.A. and Kramer, R.M. (1973) The effect of acute or chronic ether stress on plasma ACTH concentration in the rat. Endocrinology 93, 1019-1024.

Cook, S.D., Devereux, C., Troiano, R., Zito, G., Hafstein, M., Lavenhar, M., Hernandez, E. and Dowling, P.C. (1987) Total lymphoid irradiation in multiple sclerosis: blood lymphocytes and clinical course. Ann.Neurol. 22, 634-638.

Correale, J., Olsson, T., Bjork, J., Smedegard, G., Hojeberg, B. and Link, H. (1991) Sulphasalazine aggrevates experimental autoimmune encephalomyelitis and

causes an increase in the number of autoreactive T cells. J.Neuroimmunol. 34, 109-120.

Coutard, M. and Duval, D. (1985) Autoradiographic study of the nuclear transfer of the antiglucocorticoid compound RU 38486 in mouse tissues. J.Steroid Biochem. 23, 291-297.

Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) Identification and purification of an adrenal medullary protein (synexin) that causes calcium dependent aggregation of isolated chromaffin granules. J.Biol.Chem. 253, 2858-2866.

Creutz, C.E., Zaks, W.J., Hamman, H.C., Crane, S., Martin, W.H., Gould, K.L., Oddie, M. and Parsons, S.J. (1987) Identification of chromaffin granule-binding proteins. J.Biol.Chem. 262, 1860-1868.

Crompton, M.R., Owens, R.J., Totty, N.F., Moss, S.E., Waterfield, M.D. and Crumpton, M.J. (1988) Primary structure of the human membrane-associated Ca^{2+}-binding protein p68: a novel member of a protein family. EMBO J. 7, 21-27.

Crumpton, M.J. and Dedman, J.R. (1990) Protein terminology tangle. Nature 345, 212.

Cuzner, M.L. and Davison, A.N. (1979) The scientific basis of multiple sclerosis. Molec.Aspects Med. 2, 147-248.

Cuzner, M.L. (1980) Recent biochemical and immunological observations in multiple sclerosis. Neuropathol.Appl.Neurobiol. 6, 405-414.

D'Agostino, J., Vaeth, G.F. and Henning, S.J. (1982) Diurnal rhythm of total and free concentrations of serum corticosterone in the rat. Acta Endocrinol. 100, 85-90.

Daniel, P.M., Lam, D.K.C. and Pratt, O.E. (1981) Changes in the effectiveness of the blood-brain and blood-spinal cord barriers in experimental allergic encephalomyelitis. J.Neurol.Sci. 52, 211-219.

Danon, A. and Assouline, G. (1978) Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. Nature 273, 552-554.

Dau, P.C., Johnson, K.P. and Spitler, L.E. (1976) The effect of levamisole on cellular immunity in multiple sclerosis. Clin.Exp.Immunol. 226, 302-309.

Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R. (1987) Inhibition of phospholipase A_2 by "lipocortins" and calpactins. An effect of binding to substrate phospholipids. J.Biol.Chem. 262, 1698-1705.

Davidson, F.F. and Dennis, E.A. (1989) Biological relevance of lipocortins and related proteins as inhibitors of phospholipase A_2. Biochem.Pharm. 38, 3645-3651.

Davidson, J., Flower, R.J., Milton, A.S., Peers, S.H. and Rotondo, D. (1991) Antipyretic actions of human recombinant lipocortin-1. Br.J.Pharmacol. 102, 7-9.

Davies, A.A., Wigglesworth, N.M., Allen, D., Owens, R.J. and Crumpton, M.J. (1984) Nonidet P-40 extraction of lymphocyte plasma-membranes. Characterization of the insoluable residue. Biochem.J. 219, 301-308.

Davis, F.A. and Stefoski, D. (1988) Is steroid therapy in multiple sclerosis superior to corticotrophin therapy. Arch.Neurol. 45, 1180.

Davis, P. (1985) Macrophages as effector cells. Federation Proc. 44, 2925-2926.

De Blas, A.L. and Cherwinski, H.M. (1983) Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies. Anal.Biochem. 133, 214-219.

De Vellis, J., Wu, D.K. and Kumar, S. (1986) Enzyme induction and regulation of protein synthesis. In: Fedoroff, S. and Vernadakis, A. (eds.) Astrocytes: Biochemistry, physiology and pharmacology of astrocytes. Vol. 2, Academic Press, Orlando, Florida, pp. 209-237.

Di Rosa, M. and Persico, P. (1979) Mechanism of inhibition of prostaglandin biosynthesis by hydrocortisone in rat leucocytes. Br.J.Pharmacol. 66, 161-163.

Di Rosa, M., Flower, R.J., Hirata, F., Parente, L. and Russo-Marie, F. (1984) Nomenclature announcement. Anti-phospholipase proteins. Prostaglandins 28, 441-442.

Diaz-Munoz, M., Hamilton, S.L., Kaetzel, M.A., Hazarika, P. and Dedman, J.R. (1990) Modulation of Ca^{2+} release channel activity from sarcoplasmic reticulum by annexin VI (67-kDa calcimedin). J.Biol.Chem. 265, 15894-15899.

Donato, R., Giambanco, I., Pula, G. and Bianchi, R. (1990) Two novel brain proteins, CaBP33 and CaBP37 are calcium-dependent, phospholipid- and membrane-binding proteins. FEBS Lett. 262, 72-76.

Driscoll, B.F., Kies, M.W. and Alvord, E.C. (1979) Transfer of experimental allergic encephalomyelitis with guinea-pig peritoneal exudate cells. Science 203, 547-548.

Drust, D.S. and Creutz, C.E. (1988) Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature 331, 88-91.

Duncan, G.S., Peers, S.H., Carey, F., Forder, R.A. and Flower, R.J. (1993) The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin 1. Br.J.Pharmacol. 108, 62-65.

Durelli, L., Cocito, D., Riccio, A., Barile, C., Bergamasco, B., Baggio, G.F., Perla, F., Delsedime, M., Gusmaroli, G. and Bergamini, L. (1986) High-dose intravenous methylprednisolone in the treatment of multiple sclerosis: clinical-immunologic correlations. Neurology 36, 238-243.

Ebers, G.C., Bulman, D.E. and Sadovnick, A.D. (1986) A population based study of multiple sclerosis in twins. N.Engl.J.Med. 315, 1638-1642.

Einstein, E.R., Csejtey, J., Dalal, K.B., Adams, C.W.M., Bayliss, O.B. and Hallpike, J.F. (1972) Proteolytic activity and basic protein loss in and around multiple sclerosis plaques: combined biochemical and histochemical observations. J.Neurochem. 19, 653-662.

Elderfield, A-J., Newcombe, J., Bolton, C. and Flower, R.J. (1992) Lipocortins (Annexins) 1, 2, 4 and 5 are increased in the central nervous system in multiple sclerosis. J.Neuroimmunol. 39, 91-100.

Elderfield, A-J., Bolton, C. and Flower, R.J. (1993) Lipocortin 1 (annexin 1) immunoreactivity in the cervical spinal cord of Lewis rats with acute experimental allergic encephalomyelitis. J.Neurol.Sci. 119, 146-153.

Emilie, D., Galanaud, P., Baulieu, E.E. and Dormont, J. (1984) Inhibition of *in vitro* immunosuppressive effects of glucocorticosteroids by a competitive antagonist RU-486. Immunol.Lett. 8, 183-186.

Errasfa, M., Rothhut, B., Fradin, A., Billardon, C., Junien, J-L., Bure, J. and Russo-Marie, F. (1985) The presence of lipocortin in human embryonic skin fibroblasts and its regulation by anti-inflammatory steroids. Biochim.Biophys.Acta. 847, 247-254.

Errasfa, M., Bachelet, M. and Russo-Marie, F. (1988) Inhibition of phospholipase A_2 activity of guinea-pig alveolar macrophages by lipocortin-like proteins purified from mice lung. Biochem.Biophys.Res.Comm. 153, 1267-1270.

Errasfa, M. and Russo-Marie, F. (1989) A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids *in vivo* in mice. Br.J.Pharmacol. 97, 1051-1058.

Fauci, A.S. (1979) Immunosuppressive and anti-inflammatory effects of glucocorticoids. Monogr.Endocrinol. 12, 449-465.

Fava, R.A. and Cohen, S. (1984) Isolation of a calcium-dependent 35-kilodalton substrate for the epidermal growth factor receptor/kinase from A-431 cells. J.Biol.Chem. 259, 2636-2645.

Fava, R.A., McKanna, J. and Cohen, S. (1989) Lipocortin I (p35) is abundant in a restricted number of differentiated cell types in adult organs. J.Cell.Physiol. 141, 284-293.

Field, E.J. and Miller, H. (1962) Experimental allergic encephalomyelitis: comparison of protective effects of prednisolone and corticotrophin. Br.Med.J. 1, 843-844.

Flecknell, P.A. (1987) Laboratory animal anaesthesia. An introduction for research workers and technicians, Academic Press, San Diego, pp. 130.

Flower, R.J., Gryglewski, R., Herbacyznska-Cedro, K. and Vane, J.R. (1972) The effects of anti-inflammatory drugs on prostaglandin biosynthesis. Nature 238, 104-106.

Flower, R.J. and Blackwell, G.J. (1979) Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. Nature 278, 456-459.

Flower, R.J. (1984) Macrocortin and the anti-phospholipase proteins. In: Weissmann, G. (ed.) Advances in Inflammation Research. Vol. 8, Raven Press, New York, pp. 1-34.

Flower, R.J., Parente, L., Persico, P. and Salmon, J.A. (1986) A comparison of the acute inflammatory response in adrenalectomised and sham-operated rats. Br.J.Pharmacol. 87, 57-62.

Flower, R.J. (1988) Lipocortin and the mechanism of action of the glucocorticoids. Br.J.Pharmacol. 94, 987-1015.

Flower, R.J. and Dale, M.M. (1989) The anti-inflammatory effects of corticosteroids. In: Dale, M.M. and Foreman, J.C. (eds.) Textbook of immunopharmacology, Blackwell Scientific Publications, Oxford, Ed. 2nd pp. 275-289.

Fog, T. (1965) The long-term treatment of multiple sclerosis with corticoids. Acta Neurol.Scand. 41, 473-484.

Fontana, A., Kristensen, F., Dubs, R., Gemsa, D. and Weber, E. (1982) Production of prostaglandin E and interleukin-1 like factor by cultured astrocytes and C_6 glioma cells. J.Immunol. 129, 2413-2419.

Fontana, A., Fierz, W. and Wekerle, H. (1984) Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 307, 273-276.

Fradin, A., Rothhut, B., Poincelot-Canton, B., Errasfa, M. and Russo-Marie, F. (1988) Inhibition of eicosanoid and PAF formation by dexamethasone in rat inflammatory polymorphonuclear neutrophils may implicate lipocortin's'. Biochim.Biophys.Acta. 963, 248-257.

Franklin, G.M., Nelson, L.M., Heaton, R.K., Burks, J.S. and Thompson, D.S. (1988) Stress and its relationship to acute exacerbations in multiple sclerosis. Neurology 38 (suppl. 1), 254.

Frazer, H.E. and Wisdom, G.B. (1985) Detection of autoantigens by immunoblotting using a peroxidase-anti-peroxidase complex. J.Immunol.Methods 80, 221-225.

Frei, K. and Fontana, A. (1989) Immune regulatory functions of astrocytes and microglial cells within the central nervous system. In: Neuroimmune networks: Physiology and diseases, Alan R.Liss, New York, pp. 127-136.

Fretland, D.J., Widomski, D.L., Shone, R.L., Levin, S. and Gaginella, T.S. (1991) Effect of the leukotriene B_4 receptor antagonist, SC-41930, on experimental allergic encephalomyelitis (EAE) in the guinea pig. Agents Actions 34, 172-174.

Freund, J., Stern, E.A. and Pisani, T.M. (1947) Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion. J.Immunol. 57, 179-194.

Frey, B.M., Frey, F.J., Lingappa, V.R. and Trachsel, H. (1991) Expression of human recombinant lipocortin I in a wheat-germ cell-free system and *Xenopus oocytes*. Lipocortin is not secreted. Biochem.J. 275, 219-225.

Fujimoto, M., Sakata, T., Tsuruta, Y., Iwagami, S., Teraoka, H., Mihara, S-I., Fukiishi, Y. and Ide, M. (1990) Enhancement of bradykinin-induced prostacyclin synthesis in porcine aortic endothelial cells by pertusis toxin. Possible implications of lipocortin I. Biochem.Pharmacol. 40(12), 2661-2670.

Funakoshi, T., Heimark, R.L., Hendrikson, L.E., McMullen, B.A. and Fujikawa, K. (1987a) Human placental anticoagulant protein: isolation and characterization. Biochemistry 26, 5572-5578.

Funakoshi, T., Hendrikson, L.E., McMullen, B. and Fujikawa, K. (1987b) Primary structure of human placental anticoagulant protein. Biochemistry 26, 8087-8092.

Gagne, D., Pons, M. and Philibert, D. (1985) RU 38486: A potent anti-glucocorticoid *in vitro* and *in vivo*. J.Steroid Biochem. 23, 247-251.

Gaillard, R.C., Riondel, A., Muller, A.F., Hermann, W. and Baulieu, E.E. (1984) RU 486: A steroid with antiglucocorticosteroid activity that only disinhibits the human pituitary-adrenal system at a specific time of day. Proc.Natl.Acad.Sci.USA 81, 3879-3882.

Garcia-Reyes, J.A., Jenkins, D., Forsham, P.H. and Thorn, G.W. (1952) Adrenocortical function in multiple sclerosis. Arch.Neurol.Psychiat. 68, 776-782.

Gebicke-Haerter, P.J., Seregi, A., Schobert, A. and Hertting, G. (1988) Involvement of protein kinase C in prostaglandin D_2 synthesis by cultured astrocytes. Neurochem.Int. 13, 475-480.

Gebicke-Haerter, P.J., Schobert, A., Dieter, P., Honegger, P. and Hertting, G. (1991) Regulation and glucocorticoid-independent induction of lipocortin I in cultured astrocytes. J.Neurochem. 57, 175-183.

Geczy, C.L. (1984) The role of lymphokines in delayed-type hypersensitivity reactions. Immunopathol. 7, 321-346.

Geisow, M., Childs, J., Dash, B., Harris, A., Panayotou, G., Sudhof, T. and Walker, J.H. (1984) Cellular distribution of three mammalian Ca^{2+}-binding proteins related to *Torpedo* calelectrin. EMBO J. 3, 2969-2974.

Geisow, M.J. (1986) Common domain-structure of Ca^{2+} and lipid-binding proteins. FEBS Lett. 203, 99-103.

Geisow, M.J., Fritsch, U., Hexham, J.M., Dash, B. and Johnson, T. (1986) A consensus amino acid sequence repeat in *Torpedo* and mammalian Ca^{2+}-dependent membrane-binding proteins. Nature 320, 636-638.

Geisow, M.J. and Walker, J.H. (1986) New proteins involved in cell regulation by Ca^{2+} and phospholipids. Trends Biochem.Sci. 11, 420-423.

Gerke, V. and Weber, K. (1984) Identity of p36k phosphorylated upon Rous Sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin. EMBO J. 3, 227-233.

Ghiara, P., Meli, R., Parente, L. and Persico, P. (1984) Distinct inhibition of membrane-bound and lysosomal phospholipase A_2 by glucocorticoid-induced proteins. Biochem.Pharmacol. 33, 1445-1450.

Giambanco, I., Pula, G., Ceccarelli, P., Bianchi, R. and Donato, R. (1991) Immunohistochemical localization of annexin V (CaBP33) in rat organs. J.Histochem.Cytochem. 39, 1189-1198.

Giulian, D. and Lachman, L.B. (1985) Interleukin-1 stimulation of astroglial proliferation after brain injury. Science 228, 497-499.

Giulian, D., Woodward, J., Young, D.G., Krebs, J.F. and Lachman, L.B. (1988) Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J.Neurosci. 8, 2485-2490.

Glenney, J.R. and Tack, B.F. (1985) Amino-terminal sequence of p36 and associated p10: identification of the site of tyrosine phosphorylation and homology with S-100. Proc.Natl.Acad.Sci.USA 82, 7884-7888.

Glenney, J.R. (1986) Two related but different forms of the 36,000 Mr tyrosine kinase substrate (calpactins) which interact with phospholipid and actin in a Ca^{2+}-dependent manner. Proc.Natl.Acad.Sci.USA 83, 4258-4262.

Glenney, J.R., Tack, B. and Powell, M.A. (1987) Calpactins: Two distinct Ca^{2+}-regulated phospholipid- and actin-binding proteins isolated from lung and placenta. J.Cell Biol. 104, 503-511.

Goas, J.Y., Marion, J.L. and Missoum, A. (1983) High dose intravenous methylprednisolone in acute exacerbations of multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 46, 99.

Gonatas, N.K. and Howard, J.C. (1974) Inhibition of experimental allergic encephalomyelitis in rats severely depleted of T cells. Science 186, 839-841.

Goodkin, D.E., Plencher, St., Palmer-Saxerud, J., Teetzen, M. and Henstgaard, D. (1987) Cyclophosphamide in chronic progressive multiple sclerosis. Arch.Neurol. 44, 823-827.

Gould, K.L., Cooper, J.A. and Hunter, T. (1984) The 46,000-dalton tyrosine protein kinase substrate is widespread, whereas the 36,000-dalton substrate is only expressed at high levels in certain rodent tissues. J.Cell Biol. 98, 487-497.

Goulding, N.J. and Guyre, P.M. (1988) Impairment of erythrocyte-antibody rosette formation by human lipocortin 1 on human leukocytes. Biochem.Trans. 16, 730-731.

Goulding, N.J., Godolphin, J.L., Sharland, P.R., Peers, S.H., Sampson, M., Maddison, P.J. and Flower, R.J. (1990) Anti-inflammatory lipocortin 1 production by peripheral blood leukocytes in response to hydrocortisone. Lancet 335, 1416-1418.

Goulding, N.J. and Guyre, P.M. (1992) Regulation of inflammation by lipocortin 1. Immunol.Today 13, 295-297.

Grant, I., Brown, G.W., Harris, T., McDonald, W.I., Patterson, T. and Trimble, M.R. (1989) Severely threatening events and marked life difficulties preceding onset or exacerbation of multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 52, 8-13.

Grasso, R.J. (1976) Transient inhibition of cell proliferation in rat glioma monolayer cultures by cortisol. Cancer Res. 36, 2408-2414.

Graves, M.C. (1984) Viruses and demyelinating disease. pp. 517-20. In: Ellison, G. W. moderator, Multiple Sclerosis. Ann.Intern.Med. 101, 514-526.

Greenberg, M.E. and Edelman, G.M. (1983) The 34 kd pp60^{src} substrate is located at the inner surface of the plasma membrane. Cell 33, 767-779.

Greig, M.E., Gibbons, A.J. and Elliott, G.A. (1970) A comparison of the effects of melengestrol acetate and hydrocortisone acetate on experimental allergic encephalomyelitis in rats. J.Pharmacol.Exp.Ther. 173, 85-93.

Gryglewski, R.J., Panczenko, B., Korbut, R., Grodzinska, L. and Ocetkiewicz, A. (1975) Corticosteroids inhibit prostaglandin release from perfused mesenteric blood vessels of rabbit and from perfused lungs of sensitized guinea-pigs. Prostaglandins 10, 343-355.

Gupta, C., Katsumata, M., Goldman, A.S., Piddington, R. and Herold, R. (1984) Glucocorticoid-induced phospholipase A_2 inhibitory proteins mediate glucocorticoid teratogenicity *in vitro*. Proc.Natl.Acad.Sci.USA 81, 1140-1143.

Gurpide, E., Markiewicz, L., Schatz, F. and Hirata, F. (1986) Lipocortin output by human endometrium *in vitro*. J.Clin.Endocrinol.Metab. 63, 162-166.

Guseo, A. and Jellinger, K. (1975) The significance of perivascular infiltrations in multiple sclerosis. J.Neurol. 211, 51-60.

Haigler, H.T., Schlaepfer, D.D. and Burgess, W.H. (1987) Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A_2 inhibitors. J.Biol.Chem. 262, 6921-6930.

Hattori, T., Hirata, F., Hoffman, T., Hizuta, A. and Herbermann, R.B. (1983a) Inhibition of human natural killer (NK) activity and antibody dependent cellular cytotoxicity (ADCC) by lipomodulin, a phospholipase inhibitory protein. J.Immunol. 131, 662-665.

Hattori, T., Hoffman, T. and Hirata, F. (1983b) Differentiation of a histiocytic cell line by lipomodulin, a phospholipase inhibitory protein. Biochem.Biophys.Res.Comm. 111, 551-559.

Hauser, S.L., Dawson, D.M., Lehrich, J.R., Beal, M.F., Kevy, S.V., Propper, R.D., Mills, J.A. and Weiner, H.L. (1983) Intensive immunosuppression in progressive multiple sclerosis. New.Engl.J.Med. 308, 173-180.

Healy, D.L., Chrousos, G.P., Schulte, H.M., Williams, R.F., Gold, P.W., Baulieu, E.E. and Hodgen, G.D. (1983) Pituitary and adrenal responses to the antiprogesterone and antiglucocorticoid steroid RU 486 in primates. J.Clin.Endocrinol.Metab. 57, 863-865.

Hedner, P. and Rerup, C. (1960) The effect of pentobarbital (mebumal, NFN) and ether anaesthesia on the content of corticotropin in the pituitary glands of three animal species. Acta Pharmacol.Toxicol. 16, 223-228.

Hench, P.S., Kendall, E.C., Slocumb, C.H. and Polley, H.F. (1949) The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis. Proc.Staff Meet.Mayo Clin.,Rochester 25, 81.

Herbaczynska-Cedro, K. and Staszewska-Barczak, J. (1974) Adrenocortical hormones and the release of prostaglandin-like substances (PGS). II Congress of Hungarian Pharmacological Society, Budapest, 19.

Hickey, W.F., Osborn, J.P. and Kirby, W.M. (1985) Expression of Ia molecules by astrocytes during experimental allergic encephalomyelitis in the Lewis rat. Cell.Immunol. 91, 528-535.

Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1980) A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc.Natl.Acad.Sci.USA 77, 2533-2536.

Hirata, F. (1981) The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. J.Biol.Chem. 256, 7730-7733.

Hirata, F., Del Carmine, R., Nelson, C.A., Axelrod, J., Schiffmann, E., Warabi, A., De Blas, A.L., Nirendberg, M., Manganiello, V., Vaughan, M., Kumagai, S., Green, I., Decker, J.L. and Steinberg, A.D. (1981) Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proc.Natl.Acad.Sci.USA 78, 3190-3194.

Hirata, F., Notsu, Y., Iwata, M., Parente, L., Di Rosa, M. and Flower, R.J. (1982) Identification of several species of phospholipase inhibitory protein(s) by radioimmunoassay for lipomodulin. Biochem.Biophys.Res.Comm. 109, 223-230.

Hirata, F. (1983) Lipomodulin: a possible mediator of the action of glucocorticoids. In: Samuelsson, B., Paoletti, R. and Ramwell, P. (eds.) Advances

in prostaglandin, thromboxane and leukotriene research, Raven Press, New York, pp. 73-78.

Hirata, F. and Iwata, M. (1983) Role of lipomodulin, a phospholipase inhibitory protein, in immunoregulation by thymocytes. J.Immunol. 130, 1930-1936.

Hirata, F. (1984) Roles of lipomodulin: a phospholipase inhibitory protein in immunoregulation. Adv.Inflammation Res. 7, 71-78.

Hirata, F. (1989) The role of lipocortins in cellular function as a second messenger of glucocorticoids. In: Schleimer, R.P., Claman, N.H. and Oronsky, A. (eds.) Anti-inflammatory steroid action. Basic and clinical aspects, Academic Press, London, pp. 67-95.

Hirschelmann, R., Klingner, E., Schmidt, K. and Bekemeier, H. (1988a) Dexamethasone antagonism by RU 38486 in inflammatory reactions of the rat and mouse. Part 1: Degree of inflammation. Pharmazie 43, 219-220.

Hirschelmann, R., Schade, R., Klingner, E. and Bekemeier, H. (1988b) Dexamethasone antagonism by RU 38486 in inflammatory reactions of the rat. Part 2: Biochemical parameters: RNA content of inflammation cells and acute phase reactants of the blood. Pharmazie 43, 370-371.

Hong, S.C. and Levine, L. (1976) Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory steroids. Proc.Natl.Acad.Sci.USA 73, 1730-1734.

Howell, M.D., Winters, S.T., Olee, T., Powell, H.C., Carlo, D.J. and Brostoff, S.W. (1989) Vaccination against experimental allergic encephalomyelitis with T cell receptor peptides. Science 246, 668-670.

Huang, K.-S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E. and Pepinsky, R.B. (1986) Two human 35 Kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. Cell 46, 191-199.

Huang, K.-S., McGray, P., Mattaliano, R.J., Burne, C., Chow, E.P., Sinclair, L.K. and Pepinsky, R.B. (1987) Purification and characterization of proteolytic fragments of lipocortin I that inhibit phospholipase A_2. J.Biol.Chem. 262, 7639-7645.

Hullin, F., Raynal, P., Ragab-Thomas, J.M.F., Fauvel, J. and Chap, H. (1989) Effect of dexamethasone on prostaglandin synthesis and on lipocortin status in human endothelial cells. J.Biol.Chem. 264(6), 3506-3513.

Humes, J.L., Bonney, R.J., Pelus, L., Dahlgren, M.E., Sadowski, S.J., Kuehl, F.A. and Davis, P. (1977) Macrophages synthesise and release prostaglandins in response to inflammatory stimuli. Nature 269, 149-151.

Ialenti, A., Doyle, P.M., Hardy, G.N., Simpkin, D.S.E. and Di Rosa, M. (1990) Anti-inflammatory effects of vasocortin and nonapeptide fragments of uteroglobin and lipocortin I (antiflammins). Agents Actions 29, 48-49.

Ikebuchi, N.W. and Waisman, D.M. (1990) Calcium-dependent regulation of actin filament bundling by lipocortin-85. J.Biol.Chem. 265, 3392-3400.

Inamura, N., Hashimoto, M., Nakahara, K., Nakajima, Y., Nishio, M., Aoki, H., Yamaguchi, I. and Kohsaka, M. (1988) Immunosuppressive effect of FK506 on experimental allergic encephalomyelitis in rats. Int.J.Immunopharmacol. 10, 991-995.

Isacke, C.M., Lindberg, R.A. and Hunter, T. (1989) Synthesis of p36 and p35 is increased when U-937 cells differentiate in culture but expression is not inducible by glucocorticoids. Mol.Cell.Biol. 9, 232-240.

Iwasaki, A., Suda, M., Nakao, H., Nagoya, T., Saino, Y., Arai, K., Mizoguchi, T., Sato, F., Yoshizaki, H., Hirata, M., Myata, T., Shidara, Y., Murata, M. and Maki, M. (1987) Structure and expression of cDNA for an inhibitor of blood-coagulation isolated from human placenta - a new lipocortin-like protein. J.Biochem.(Tokyo) 102, 1261-1273.

Jacobs, L., Salazar, A.M., Herndon, R., Reese, P.A., Freeman, A., Jozefowicz, R., Cuetter, A., Husain, F., Smith, W.A., Ekes, R. and O'Malley, J.A. (1987) Intrathecally administered natural human fibroblast interferon reduces exacerbations of multiple sclerosis. Results of a multicentre double-blinded study. Arch.Neurol. 44, 589-595.

Jaquot, J., Dupuit, F., Elbtaouri, H., Hinnrasky, J., Antonicelli, F., Haye, B. and Puchelle, E. (1990) Production of lipocortin-like proteins by cultured human tracheal submucosal gland cells. FEBS Lett. 274, 131-135.

Jellinger, K. (1977) Inflammatory lesions in multiple sclerosis. In: Delmotte, P., Hommes, O.R. and Gonsette, R. (eds.) Immunosuppressive therapy in multiple sclerosis, European Press, Gent, pp. 164-180.

Johnson, M.D., Kamso-Pratt, J., Pepinsky, R.B. and Whetsell Jr., W.O. (1989a) Lipocortin-1 immunoreactivity in central and peripheral nervous system glial tumours. Hum.Pathol. 20, 772-776.

Johnson, M.D., Kamso-Pratt, J.M., Whetsell Jr., W.O. and Pepinsky, R.B. (1989b) Lipocortin-1 immunoreactivity in the normal human central nervous system and lesions with astrocytosis. Am.J.Clin.Pathol. 92, 424-429.

Johnson, R.T., Katzmann, R., McGeer, E., Price, D., Shooter, E.M. and Silberberg, D. (1979) Report of the panel on inflammatory, demyelinating and degenerative diseases. NIH Publ. No. 79-1916, US Department of Health, Education and Welfare, Washington DC.

Johnston, P.A., Perin, M.S., Reynolds, G.A., Wasserman, S.A. and Sudhof, T.C. (1990) Two novel annexins from Drosophila melanogaster cloning, characterization and differential expression in development. J.Biol.Chem. 265, 11382-11388.

Juhler, M. (1988) Pathophysiological aspects of acute experimental allergic encephalomyelitis. Acta Neurol.Scand. 78 (suppl.), 1-21.

Kabat, E.A., Wolf, A. and Bezer, A.E. (1952) Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys. VII. The effect of cortisone. J.Immunol. 68, 265-275.

Kaplan, R., Jaye, M., Burgess, W.H., Schlaepfer, D.D. and Haigler, H.T. (1988) Cloning and expression of cDNA for human endonexin-II, a Ca^{2+} and phospholipid-binding protein. J.Biol.Chem. 263, 8037-8043.

Kappos, L. (1988) Clinical trials of immunosuppression and immunomodulation in multiple sclerosis. J.Neuroimmunol. 20, 261-268.

Kappos, L., Patzold, U., Domatsch, S., Poser, S., Haas, J., Krauseneck, P., Malin, J.P., Fierz, W., Graffenried, B.U. and Gugerli, U.S. (1988) Cyclosporin versus azathioprine in the long term treatment of multiple sclerosis. Ann.Neurol. 23, 56-63.

Kaschka, W.P. and Hilgers, R. (1980) Blood lymphocyte subpopulations show characteristic changes during ACTH therapy in acute exacerbations of multiple sclerosis. Acta Neurol.Scand. 61, 275-286.

Keller, M., Jackisch, R., Seregi, A. and Hertting, G. (1985) Comparison of prostanoid forming capacity of neuronal and astroglial cells in primary cultures. Neurochem.Int. 7, 655-665.

Kermode, A.G., Thompson, A.J,, Tofts, P., MacManus, D.G., Kendall, B.E., Kingsley, D.P.E., Moseley, I.F., Rudge, P. and McDonald, W.I. (1990) Breakdown of the blood-brain barrier precedes symptoms and other MRI signs of new lesions in multiple sclerosis: pathogenetic and clinical implications. Brain 113, 1477-1489.

Kesselring, J., Miller, D.H., MacManus, D.G., Johnson, G., Milligan, N.M., Scolding, N., Compston, D.A.S. and McDonald, W.I. (1989) Quantitative magnetic resonance imaging in multiple sclerosis: the effect of high dose intravenous methylprednisolone. J.Neurol.Neurosurg.Psychiatry 52, 14-17.

Ketelaer, Ch.J. and Delmotte, P. (1972) Results of adrenal and pituitary stimulation tests in patients with multiple sclerosis. Acta Neurol.Scand. 48, 467-478.

Kies, M.W. (1965) Chemical studies on an encephalitogenic protein from guinea pig brain. Ann.N.Y.Acad.Sci. 122, 161-169.

Killian, J.M., Bressler, R.B., Armstrong, R.M. and Huston, D.P. (1988) Controlled pilot trial of monthly intravenous cyclophosphamide in multiple sclerosis. Arch.Neurol. 45, 27-30.

Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H. (1987a) FK506, a novel immunosuppressant isolated from a streptomyces. I. Fermentation, isolation and physico-chemical and biological characteristics. J.Antibiot. 40, 1249-1255.

Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Ochiai, T. (1987b) FK506, a novel immunosuppressant isolated from a streptomyces. II. Immunosuppressive effect of FK506 in vitro. J.Antibiot. 40, 1256-1264.

Kitajima, Y., Owada, K., Mitsui, H. and Yaoita, H. (1991) Lipocortin I (annexin I) is preferentially localized on the plasma membrane in keratinocytes of psoriatic lesional epidermis as shown by immunofluorescence microscopy. J.Invest.Dermatol. 97, 1032-1038.

Klapps, P., Seyfert, S., Fischer, T. and Scherbaum, W.A. (1992) Endocrine function in multiple sclerosis. Acta Neurol.Scand. 85, 353-357.

Klee, C.B. (1988) Ca^{2+}-dependent phospholipid-(and membrane-) binding proteins. Biochemistry 27, 6645-6653.

Kniss, D.A. and Burry, R.W. (1985) Glucocorticoid hormones inhibit DNA synthesis in glial cells cultured in chemically defined medium. Exp.Cell Res. 161, 29-40.

Kolb, L.C., Karlson, A.G. and Sayre, G.P. (1952) Prevention of experimental allergic encephalomyelitis by various agents. Trans.Am.Neurol.Assoc. 77, 117-126.

Koltai, M., Tosaki, A., Adam, G., Joo, F., Nemecz, G. and Szekeres, L. (1984) Prevention by macrocortin of global cerebral ischaemia in Sprague-Dawley rats. Eur.J.Pharmacol. 105, 347-350.

Komarek, A. and Dietrich, F.M. (1971) Chemical prevention of experimental allergic encephalomyelitis in rats: A quantitative evaluation of steroids and various non-steroid drugs. Arch.Int.Pharmacod. 193, 249-257.

Koopmans, R.A., Li, D.K.B., Oger, J.J.F., Mayo, J. and Paty, D.W. (1989) The lesion of multiple sclerosis: imaging of acute and chronic stages. Neurology 39, 959-963.

Kovacic, R.T., Tizard, R., Cate, R.L., Frey, A.Z. and Wallner, B.P. (1991) Correlation of gene and protein structure of rat and human lipocortin 1. Biochemistry 30, 9015-9021.

Kretsinger, R.H. and Creutz, C.E. (1986) Consensus in exocytosis. Nature 320, 573.

Kurtzke, J.F. (1980) Epidemiologic contributions to multiple sclerosis: an overview. Neurology 30, 61-79.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lampert, P.W. and Carpenter, S. (1965) Electron microscopic studies on the vascular permeability and the mechanism of demyelination in experimental allergic encephalomyelitis. J.Neuropath.Exp.Neurol. 24, 11-24.

Learmonth, M.P., Howell, S.A., Harris, A.C.M., Ames, S.B., Patel, Y., Giambanco, I., Bianchi, R., Pula, G., Ceccarelli, P., Donato, R., Green, B.N. and Aitken, A. (1992) Novel isoforms of CaBP33/37 (annexin V) from mammalian brain: Structural and phosphorylation differences that suggest distinct biological roles. Biochim.Biophys.Acta Protein Struct.Mol.Enzymol. 1160, 76-83.

Lee, S.C., Moore, G.R.W., Golensky, G. and Raine, C.S. (1990) Multiple sclerosis: A role for astroglia in active demyelination suggested by Class II MHC expression and ultrastructural study. J.Neuropathol.Exp.Neurol. 49, 122-136.

Leibowitz, S. and Kennedy, L. (1972) Cerebral vascular permeability and cellular infiltration in experimental allergic encephalomyelitis. Immunol. 22, 859-869.

Lelievre, V., Martin, B., Junien, J.L. and Bure, L. (1988) Local anti-inflammatory activities of tixocortol 21-pivalate, inhibition of prostaglandins and leukotrienes synthesis in carrageenin-induced pleurisy. Reversion of effects by RU 486. Agents Actions 24, 172-178.

Leonard, J.P., Mackenzie, F.J., Patel, H.A. and Cuzner, M.L. (1990) Splenic noradrenergic and adrenocortical reponses during the preclinical and clinical stages of adoptively transferred experimental autoimmune encephalomyelitis (EAE). J.Neuroimmunol. 26, 183-186.

Leutz, A. and Schachner, M. (1981) Epidermal growth factor stimulates DNA-synthesis of astrocytes in primary cerebellar cultures. Cell Tissue Res. 220, 393-404.

Levi-Strauss, M. and Mallat, M. (1987) Primary cultures of murine astrocytes produce C3 and factor B, two components of the alternative pathway of complement activation. J.Immunol. 139, 2361-2366.

Levine, S., Strebel, R., Wenk, E.J. and Harman, P.J. (1962a) Suppression of experimental allergic encephalomyelitis by stress. Proc.Soc.Exp.Biol.Med. 109, 294-298.

Levine, S., Wenk, E.J., Muldoon, T.N. and Cohen, S.G. (1962b) Enhancement of experimental allergic encephalomyelitis by adrenalectomy. Proc.Soc.Exp.Biol.Med. 111, 383-385.

Levine, S. and Wenk, E.J. (1963) Encephalitogenic potentcies of nervous system tissues. Proc.Soc.Exp.Biol.Med. 114, 220-224.

Levine, S., Simon, J. and Wenk, E.J. (1966) Edema of the spinal cord in experimental allergic encephalomyelitis. Proc.Soc.Exp.Biol.Med. 123, 539-541.

Levine, S. and Strebel, R. (1969) Allergic encephalomyelitis: inhibition of cellular passive transfer by exogenous and endogenous steroids. Experientia 25, 189-190.

Levine, S. and Sowinski, R. (1980) Therapy of allergic encephalomyelitis in rats after onset of paralysis. In: Davison, A.N. and Cuzner, M.L. (eds.) The suppression of experimental allergic encephalomyelitis and multiple sclerosis, Academic Press, London, pp. 199-209.

Levine, S., Sowinski, R. and Steiner, B. (1980) Effects of experimental allergic encephalomyelitis on thymus and adrenal in relation to remission and relapse. Proc.Soc.Exp.Biol.Med. 165, 218-224.

Lewis, G.P. and Piper, P.J. (1975) Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids. Nature 254, 308-311.

Libermann, T.A., Razon, N., Bartal, A.D., Yarden, Y., Schlessinger, J. and Soreq, H. (1984) Expression of epidermal growth factor receptors in human brain tumours. Cancer Res. 44, 753-760.

Liebowitz, S. (1983) The immunology of multiple sclerosis. In: Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W. (eds.) Multiple sclerosis: pathology diagnosis and management, Chapman and Hall, London, pp. 379-412.

Likosky, W.H. (1988) Experience with cyclophosphamide in multiple sclerosis: the cons. Neurology 38 (suppl. 2), 14-18.

Lippman, M. and Barr, R. (1977) Glucocorticoid receptors in purified subpopulations of human peripheral blood lymphocytes. J.Immunol. 118, 1977-1981.

Long, J.B. and Holaday, J.W. (1985) Blood-brain barrier: endogenous modulation by adrenal-cortical function. Science 227, 1580-1582.

Lumsden, C.E. (1970) The neuropathology of multiple sclerosis. In: Vinken, P.J. and Bruyn, G.W. (eds.) Handbook of clinical neurology, North-Holland, Amsterdam, pp. 217-309.

Lundgren, J.D., Hirata, F., Marom, Z., Logun, C., Steel, L., Kaliner, M. and Shelhamer, J. (1988) Dexamethasone inhibits respiratory glycoconjugate secretion from feline airways *in vitro* by the induction of lipocortin (lipomodulin) synthesis. Am.Rev.Respir.Dis. 137, 353-357.

Lyons, P.R., Newman, P.K. and Saunders, M. (1988) Methylprednisolone therapy in multiple sclerosis: a profile of adverse effects. J.Neurol.Neurosurg.Psychiatry 51, 285-287.

Mackenzie, F.J., Leonard, J.P. and Cuzner, M.L. (1989) Changes in lymphocyte β-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat. J.Neuroimmunol. 23, 93-100.

MacPhee, I.A.M., Antoni, F.A. and Mason, D.W. (1989) Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J.Exp.Med. 169, 431-445.

Maida, E. and Summer, K. (1979) Serum cortisol levels of multiple sclerosis patients during ACTH treatment. J.Neurol. 220, 143-148.

Maridonneau-Parini, I., Errasfa, M. and Russo-Marie, F. (1989) Inhibition of O_2^- generation by dexamethasone is mimicked by lipocortin I in alveolar macrophages. J.Clin.Invest. 83, 1936-1940.

Marki, F., Pfeilschifter, J., Rink, H. and Wiesenberg, I. (1990) "Antiflammins": Two nonapeptide fragments of uteroglobin and lipocortin I have no phospholipase A_2- inhibitory and anti-inflammatory activity. FEBS Lett. 264, 171-175.

Massey, D., Traverso, V. and Maroux, S. (1991) Lipocortin IV is a basolateral cytoskeleton constituent of rabbit enterocytes. J.Biol.Chem. 266, 3125-3130.

Matsumoto, Y., Hara, N., Tanaka, R. and Fujiwara, M. (1986) Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis, with special reference to Ia-positive cells with dendritic morphology. J.Immunol. 136, 3668-3676.

Matsumoto, Y., Hanawa, H., Tsuchida, M. and Abo, T. (1993) *In situ* inactivation of infiltrating T cells in the central nervous system with autoimmune encephalomyelitis. The role of astrocytes. Immunol. 79, 381-390.

Mauch, E., Kornhuber, H.H., Pfrommer, U., Hahnel, A., Laufen, H. and Krapf, H. (1989) Effective treatment of chronically progressive multiple sclerosis with low-dose cyclophosphamide with minor side-effects. Eur.Arch.Psychiatr.Neurol.Sci. 238, 115-117.

Maurer-Fogy, I., Reutelingsperger, C.P.M., Pieters, J., Bodo, G., Stratowa, C. and Hauptmann, R. (1988) Cloning and expression of cDNA for human vascular anticoagulant, a Ca^{2+} dependent phospholipid binding protein. Eur.J.Biochem. 174, 585-592.

McDonald, W.I. and Halliday, A.M. (1977) Diagnosis and classification of multiple sclerosis. Br.Med.Bull. 33, 4-8.

McDonald, W.I. and Barnes, D. (1989) Lessons from magnetic resonance imaging in multiple sclerosis. TINS 12, 376-379.

McFarlin, D.E. and McFarland, H.F. (1982a) Multiple sclerosis. Part 1. New.Engl.J.Med. 307, 1183-1188.

McFarlin, D.E. and McFarland, H.F. (1982b) Multiple sclerosis. Part 2. New.Engl.J.Med. 307, 1246-1251.

McKhann, G.M. (1982) Multiple sclerosis. Ann.Rev.Neurosci. 5, 219-239.

Merritt, H.H., Glaser, G.H. and Herrmann, C. (1954) A study of the short- and long-term effects of adrenal steroids in the clinical patterns of multiple sclerosis. Ann.N.Y.Acad.Sci. 58, 625-632.

Miele, L., Cordella-Miele, E., Facchiano, A. and Mukherjee, A.B. (1988) Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin I. Nature 335, 726-730.

Millac, P., Cook, D.B. and Chase, K. (1969) Endocrine function in multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 32, 414-418.

Miller, D.H., Rudge, P., Johnson, G., Kendall, B.E., MacManus, D.G., Moseley, I.F., Barnes, D. and McDonald, W.I. (1988) Serial gadolinium enhanced magnetic resonance imaging in multiple sclerosis. Brain 111, 927-937.

Miller, H., Newell, D.J. and Ridley, A. (1961a) Multiple sclerosis: trials of maintenance treatment with prednisolone and soluable aspirin. Lancet 1, 127-129.

Miller, H., Newell, D.J. and Ridley, A. (1961b) Multiple sclerosis: treatment of acute exacerbations with corticotrophin (ACTH). Lancet 2, 1120-1122.

Miller, J.H.D., Vas, C.J., Noronha, M.J., Liversedge, L.A. and Rawson, M.D. (1967) Longterm treatment of multiple sclerosis with corticotrophin. Lancet 2, 429-431.

Milligan, N.M., Newcombe, R. and Compston, D.A.S. (1987) A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis: 1. Clinical effects. J.Neurol.Neurosurg.Psychiatry 50, 511-516.

Minagawa, H., Takenaka, A., Itoyama, Y. and Mori, R. (1987) Experimental allergic encephalomyelitis in the Lewis rat. A model of predictable relapse by cyclophosphamide. J.Neurol.Sci. 78, 225-235.

Mitchell, M.D., Lytton, F.D. and Varticovski, L. (1988) Paradoxical stimulation of both lipocortin and prostaglandin production in human amnion cells by dexamethasone. Biochem.Biophys.Res.Comm. 151, 137-141.

Mollenhauer, J. and von der Mark, K. (1983) Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes. EMBO J. 2/1, 45-50.

Moore, P.B. and Dedman, J.R. (1982) Calcium-dependent protein binding to phenothiazine columns. J.Biol.Chem. 257, 9663-9667.

Moss, S.E., Crompton, M.R., Edwards, H.C. and Crumpton, M.J. (1988) p68: a new protein of the lipocortin family. Biochem.Soc.Trans. 16, 813-814.

Moyer, A.W., Jervis, G.A., Black, J., Koprowski, H. and Cox, H.R. (1950) Action of the adrenocorticotropic hormone (ACTH) in experimental allergic encephalomyelitis of the guinea pig. Proc.Soc.Exp.Biol.Med. 75, 387-390.

Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T.A. (1990) A novel pathway for secretory proteins. Trends Biochem.Sci. 15, 86-88.

Munck, A. and Leung, K. (1977) Glucocorticoid receptors and mechanisms of action. In: Pasqualini, J.R. (ed.) Receptors and mechanism of action of steroid hormones. Part II, Marcel Dekker, New York, pp. 311-319.

Munck, A., Guyre, P.M. and Holbrook, N. (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr.Rev. 5, 25-44.

Naess, A. and Nyland, H. (1981) Effect of ACTH treatment on CSF and blood lymphocyte subpopulations in patients with multiple sclerosis. Acta Neurol.Scand. 63, 57-66.

Nathan, C.F., Murray, H.W. and Cohn, Z.A. (1982) The macrophage as an effector cell. New.Engl.J.Med. 303, 622-626.

Newcombe, J., Woodroofe, M.N. and Cuzner, M.L. (1986) Distribution of glial fibrillary acidic protein in gliosed human white matter. J.Neurochem. 47, 1713-1719.

Newcombe, J. and Cuzner, M.L. (1988) Monoclonal antibody 14E identifies the oligodendrocyte cell body in normal adult human and rat white matter. J.Neuroimmunol. 19, 11-20.

Newcombe, J., Hawkins, C.P., Henderson, C.L., Patel, H.A., Woodroofe, M.N., Hayes, G.M., Cuzner, M.L., MacManus, D., du Boulay, E.P.G.H. and McDonald, W.I. (1991) Histopathology of multiple sclerosis lesions detected by magnetic resonance imaging in unfixed postmortem central nervous system tissue. Brain 114, 1013-1023.

Nijkamp, F.P., Flower, R.J., Moncada, S. and Vane, J.R. (1976) Partial purification of RCS-RF (rabbit aorta contracting substance-releasing factor) and inhibition of its activity by anti-inflammatory steroids. Nature 263, 479-482.

Nishida, T., Nakai, S., Kawakami, T., Aihara, K., Nistino, N. and Nirai, Y. (1989) Dexamethasone regulation of the expression of cytokine mRNAs induced by interleukin-1 in the astrocytoma line U373MG. FEBS Lett. 243, 129-138.

Nisipeanu, P. and Korczyn, A.D. (1993) Psychological stress as risk factor for exacerbations in multiple sclerosis. Neurology 43, 1311-1312.

Norrby, E., Link, H., Olsson, J-E., Panelius, M., Salmi, A. and Vandvik, B. (1974) Comparison of antibodies against different viruses in cerebrospinal fluid and serum samples from patients with multiple sclerosis. Infect.Immun. 10, 688-694.

Northup, J.K., Valentine-Braun, K.A., Johnson, L.K., Severson, D.C. and Hollenberg, M.D. (1988) Evaluation of the anti-inflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J.Clin.Invest. 82, 1347-1352.

Noseworthy, J.H. (1991) Therapeutics of multiple sclerosis. Clin.Neuropharmacol. 14, 49-61.

Oger, J.J.F. and Arnason, B.G.W. (1980) HLA patterns in multiple sclerosis. In: Bauer, H.J., Poser, S. and Ritter, G. (eds.) Progress in multiple sclerosis research, Springer-Verlag, New York, pp. 460-464.

Oldendorf, W.H. and Towner, H.F. (1974) Blood-brain barrier and DNA changes during the evolution of experimental allergic encephalomyelitis. J.Neuropathol.Exp.Neurol. 33, 616-631.

Oldstone, M.B.A. and Dixon, F.J. (1968) Immunohistochemical study of allergic encephalomyelitis. Am.J.Pathol. 52, 251-263.

Ovadia, H. and Paterson, P.Y. (1982) Effect of indomethacin treatment upon actively-induced and transferred experimental allergic encephalomyelitis (EAE) in Lewis rats. Clin.Exp.Immunol. 49, 386-392.

Owhashi, M. and Heber-Katz, E. (1988) Protection from experimental allergic encephalomyelitis conferred by a monoclonal antibody directed against a shared idiotype on rat T cell receptors specific for myelin basic protein. J.Exp.Med. 168, 2153-2164.

Panitch, H.S., Hirsch, R.L., Haley, A.S. and Johnson, K.P. (1987) Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet 1, 893-895.

Pardridge, W.M. and Mietus, L.J. (1979) Transport of steroid hormones through the rat blood-brain barrier. J.Clin.Invest. 64, 145-154.

Parente, L., Di Rosa, M., Flower, R.J., Ghiara, P., Meli, R., Persico, P., Salmon, J.A. and Wood, J.N. (1984) Relationship between the anti-phospholipase and anti-inflammatory effects of glucocorticoid-induced proteins. Eur.J.Pharmacol. 99, 233-239.

Parente, L. and Flower, R.J. (1985a) Hydrocortisone and 'macrocortin' inhibit the zymosan-induced release of Lyso-PAF from rat peritoneal leucocytes. Life.Sci. 36, 1225-1231.

Parente, L. and Flower, R.J. (1985b) The generation of Lyso-PAF in experimental inflammation. In: Higgs, G.A. and Williams, T.J. (eds.) Inflammatory mediators, Macmillan, Basingstoke, pp. 65.

Parente, L., Becherucci, C., Perretti, M., Solito, E., Mugridge, K.G., Galeotti, C.L., Raugei, G., Melli, M. and Sanso, M. (1990) Are lipocortins the second messengers of the anti-inflammatory actions of glucocorticoids. In: Melli, M. and Parente, L. (eds.) Cytokines and lipocortins in inflammation and differentiation, Wiley Liss, New York, pp. 55-68.

Paterson, P.Y. (1960) Transfer of allergic encephalomyelitis in rats by means of lymph node cells. J.Exp.Med. 111, 119-136.

Paterson, P.Y. and Drobish, G.H. (1969) Cyclophosphamide: effect on experimental allergic encephalomyelitis in Lewis rats. Science 165, 191-192.

Paterson, P.Y. (1976) Experimental autoimmune (allergic) encephalomyelitis: induction pathogenesis and suppression. In: Miescher, P.A. and Muller-Eberhard, M.J. (eds.) Textbook of immunopathology, Grune and Stratton, New York, Ed. 2nd, pp. 179-213.

Paterson, P.Y. (1978) The demyelinating diseases: Clinical and experimental studies in animals and man. In: Samter, M., Alexander, N., Rose, B., Sherman, W.B., Talmage, D.W. and Vaughn, J.H. (eds.) Immunological diseases, Little Brown, Boston, Ed. 3rd pp. 1400-1435.

Paterson, P.Y. (1982) Molecular and cellular determinants of neuroimmunologic inflammatory disease. Federation Proc. 41, 2569-2576.

Patte, C., Rothhut, B., Russo-Marie, F. and Blanquet, P.R. (1991) Possible involvement of lipocortin in the initiation of DNA synthesis by human endothelial cells. Exp.Cell Res. 197, 12-20.

Pearson, C.M. (1956) Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proc.Soc.Exp.Biol.Med. 91, 95-101.

Peers, S.H,, Moon, D. and Flower, R.J. (1988) Reversal of the anti-inflammatory effects of dexamethasone by the glucocorticoid antagonist RU 38486. Biochem.Pharmacol. 37, 556-557.

Peers, S.H., Taylor, R.D. and Flower, R.J. (1987) A novel binding assay for phospholipase A_2. Biochem.Pharmacol. 36, 4287-4291.

Peers, S.H., Smillie, F., Elderfield, A.J. and Flower, R.J. (1993) Glucocorticoid- and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes *in vivo*. Br.J.Pharmacol. 108, 66-72.

Pender, M.P. (1989) Recovery from acute experimental allergic encephalomyelitis in the Lewis rat. Early restoration of nerve conduction and repair by schwann cells and oligodendrocytes. Brain 112, 393-416.

Pepinsky, R.B. and Sinclair, L.K. (1986) Epidermal growth factor-dependent phosphorylation of lipocortin. Nature 321, 81-84.

Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Fabel, T., Ribolini, A., Garwin, J.L. and Wallner, B.P. (1986) Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A_2 activity from rat peritoneal exudates. J.Biol.Chem. 261, 4239-4246.

Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Chow, E.P., Burne, C., Huang, K-S., Pratt, D., Wachter, L., Hession, C., Frey, A.Z. and Wallner, B.P. (1988) Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. J.Biol.Chem. 263(22), 10799-10811.

Pepinsky, R.B., Sinclair, L.K., Chow, E.P. and O'Brine-Greco, B. (1989) A dimeric form of lipocortin-1 in human placenta. Biochem.J. 263, 97-103.

Pepinsky, R.B., Sinclair, L.K., Dougas, I., Liang, C-M., Lawton, P. and Browning, J.L. (1990) Monoclonal antibodies to lipocortin-1 as probes for biological function. FEBS Lett. 261, 247-252.

Percy, A.K., Nobrega, F.T., Okazaki, H., Galattre, E. and Kurland, L.T. (1971) Multiple sclerosis in Rochester, Minn: a 60-year appraisal. Arch.Neurol. 25, 105-111.

Perretti, M., Becherucci, C., Mugridge, G., Solito, E., Silvestri, S. and Parente, L. (1991) A novel anti-inflammatory peptide from human lipocortin 5. Br.J.Pharmacol. 103, 1327-1332.

Perretti, M., Croxtall, J.D. and Flower, R.J. (1992) Endogenous lipocortin-1 mediates dexamethasone inhibition of interleukin-1-induced neutrophil accumulation *in vivo*. Br.J.Pharmacol. 107, 77.

Perretti, M. and Flower, R.J. (1993) Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. J.Immunol. 150, 992-999.

Pfaffle, M., Ruggiero, F., Hofmann, H., Fernandez, P., Selmin, O., Yamada, Y., Garrone, R. and von der Mark, K. (1988) Biosynthesis, secretion and extracellular localization of anchorin CII, a collagen-binding protein of the calpactin family. EMBO J. 7, 2335-2342.

Philibert, D., Deraedt, R. and Teutsch, G. (1981) RU 38486 - A potent anti-glucocorticoid *in vivo*. Presented at the VIII International Congress of Pharmacology, Tokyo, Japan, 668.

Philipps, C., Rose-John, S., Rincke, G., Furstenberger, G. and Marks, F. (1989) cDNA-cloning, sequencing and expression in glucocorticoid-stimulated quiescent swiss 3T3 fibroblasts of mouse lipocortin I. Biochem.Biophys.Res.Comm. 159, 155-162.

Piltch, A., Sun, L., Fava, R.A. and Hayashi, J. (1989) Lipocortin-independent effect of dexamethasone on phospholipase activity in a thymic epithelial cell line. Biochem.J. 261, 395-400.

Polak, J.M. and Van Noorden, S. (1986) Immunocytochemistry. Modern methods and applications, John Wright and Sons, Bristol.

Pollard, H.B., Burns, A.L. and Rojas, E. (1990) Synexin (annexin VII): a cytosolic calcium-binding protein which promotes membrane fusion and forms calcium channels in artificial bilayer and natural membranes. J.Membrane Biol. 117, 101-112.

Polman, C.H., Matthaei, I., De Groot, C.J.A., Koetsier, J.C., Sminia, T. and Dijkstra, C.D. (1988) Low-dose cyclosporin A induces relapsing remitting experimental allergic encephalomyelitis in the Lewis rat. J.Neuroimmunol. 17, 209-216.

Polman, C.H., Van der Wiel, H.E., Netelenbos, J.C., Teule, G.J.J. and Koetsier, J.C. (1991) A commentary on steroid treatment in multiple sclerosis. Arch.Neurol. 48, 1011-1012.

Poser, C.M. (1989) Corticotrophin is superior to corticosteroids in the treatment of MS. Arch.Neurol. 46, 946.

Poser, C.M. (1992) Multiple sclerosis. Observations and reflections - a personal memoir. J.Neurol.Sci. 107, 127-140.

Powell, M.A. and Glenney, J.R. (1987) Calpactin-I and calpactin-II- calcium, phospholipid and actin binding-proteins. J.Cell.Biochem. S11B, 155.

Pradel, L.A. and Rendon, A. (1993) Annexin 1 is present in different molecular forms in rat cerebral cortex. FEBS Lett. 327, 41-44.

Prineas, J.W. and Connell, F. (1978) Multiple sclerosis. Capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. Ann.Neurol. 10, 149-158.

Prineas, J.W. and Connell, F. (1979) Remyelination in multiple sclerosis. Ann.Neurol. 5, 22-31.

Prineas, J.W. and Wright, R.G. (1978) Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis. Lab.Invest. 38, 409-421.

Prineas, J.W., Kwon, E.E, Cho, E.S. and Sharer, L.R. (1984) Continual breakdown and regeneration of myelin in progressive multiple sclerosis. Ann.N.Y.Acad.Sci. 436, 11-32.

Prosiegel, M., Neu, I., Mallinger, J., Wildfeuer, A., Mehlber, L., Vogl, S., Hoffmann, G. and Ruhenstroth-Bauer, G. (1989a) Suppression of experimental autoimmune encephalomyelitis by dual cyclo-oxygenase and 5-lipoxygenase inhibition. Acta Neurol.Scand. 79, 223-226.

Prosiegel, M., Neu, I., Ruhenstroth-Bauer, G., Hoffmann, G., Vogl, S., Mehlber, L. and Wildfeuer, A. (1989b) Suppression of experimental autoimmune encephalomyelitis by sulfasalazine. New.Engl.J.Med. 321, 545-546.

Pula, G., Bianchi, R., Leccarelli, P., Giambanco, I. and Donato, R. (1990) Characterization of mammalian heart annexins with special reference to CaBP33 (annexin V). FEBS Lett. 277, 53-58.

Raine, C.S. (1984) Analysis of autoimmune demyelination: Its impact upon multiple sclerosis. Lab.Invest. 50, 608-635.

Raine, C.S. and Scheinberg, L.C. (1988) On the immunopathology of plaque development and repair in multiple sclerosis. J.Neuroimmunol. 20, 189-201.

Raynal, P., van Bergen en Henegouwen, P.M.P., Hullin, F., Ragab-Thomas, J.M.F., Fauvel, J., Verkleij, A. and Chap, H. (1992) Morphological and biochemical evidence for partial nuclear localization of annexin 1 in endothelial cells. Biochem.Biophys.Res.Commun. 186, 432-439.

Reder, A.T., Lowy, M.T., Meltzer, H.Y. and Antel, J.P. (1987) Dexamethasone suppression test abnormalities in multiple sclerosis: relation to ACTH therapy. Neurology 37, 849-853.

Reeves, S.A., Chavez-Kappel, C., Davis, R., Rosenblum, M. and Israel, M.A. (1992) Developmental regulation of annexin II (Lipocortin 2) in human brain and expression in high grade glioma. Cancer Res. 52, 6871-6876.

Regnouf, F., Rendon, A. and Pradel, L.A. (1991) Biochemical characterization of annexins I and II isolated from pig nervous tissue. J.Neurochem. 56, 1985-1996.

Relton, J.K., Strijbos, P.J.L.M., O'Shaughnessy, C.T., Carey, F., Forder, R.A., Tilders, F.J.H. and Rothwell, N.J. (1991) Lipocortin-1 is an endogenous inhibitor of ischaemic damage in the rat brain. J.Exp.Med. 174, 305-310.

Remlinger, J. (1905) Accidents paralytiques au cours du traitment anti-rabique. Ann.Inst.Pasteur 19, 625-646.

Reulen, H.J., Hadjidimos, A. and Schurmann, K. (1972) The effect of dexamethasone on water and electrolyte content and on rCBF in perifocal brain oedema in man. In: Reulen, H.J. and Schurmann, K. (eds.) Steroids and brain oedema, Springer Verlag, Berlin, pp. 239-252.

Rinne, U.K. (1968) Corticotrophin secretion capacity in multiple sclerosis. Eur.Neurol. 1, 221-233.

Rivers, T.M. and Schwentker, F.F. (1935) Encephalomyelitis accompanied by myelin distruction experimentally produced in monkeys. J.Exp.Med. 61, 689-702.

Robitzki, A., Schroder, H.C., Ugarkovic, D., Gramzow, M., Fritsche, U., Batel, R. and Muller, W.E.G. (1990) cDNA structure and expression of calpactin, a peptide involved in Ca^{2+}-dependent cell aggregation in sponges. Biochem.J. 271, 415-420.

Rodriguez, M. (1989) Multiple sclerosis: basic concepts and hypothesis. Mayo Clin.Proc. 64, 570-576.

Rojas, E. and Pollard, H.B. (1987) Membrane capacity measurements suggest a calcium-dependent insertion of synexin into phosphatidylserine bilayers. FEBS Lett. 217, 25-31.

Romisch, J., Grote, M., Weithmann, K.U., Heimburger, N. and Amann, E. (1990) Annexin proteins PP4 and PP4-X. Comparative characterization of biological activities of placental and recombinant proteins. Biochem.J. 272, 223-229.

Rose, A.S., Kuzma, J.W., Kurtzke, J.F., Namerow, N.S., Sibley, W.A. and Tourtellotte, W.W. (1970) Cooperative study in the evaluation of therapy in multiple sclerosis: ACTH vs. placebo. Final report. Neurology 20, 1-59.

Rosenthale, M.E., Datko, L.J., Kassarich, J. and Schneider, F. (1969) Chemotherapy of experimental allergic encephalomyelitis (EAE). Arch.Int.Pharmacod. 179, 251-275.

Rosnowska, M., Cendrowski, W., Sobocinska, Z. and Wieczorkiewicz, A. (1981) Prostaglandins E_2 and $F_{2\alpha}$ in the cerebrospinal fluid in patients with multiple sclerosis. Acta Med.Pol. 22, 97-103.

Ross, T.S., Tait, J.F. and Majerus, P.W. (1990) Identity of inositol 1,2-cyclic phosphate 2-phosphohydrolase with lipocortin III. Science 248, 605-607.

Rothhut, B., Russo-Marie, F., Wood, J., Di Rosa, M. and Flower, R.J. (1983) Further characterization of the glucocorticoid-induced antiphospholipase protein "renocortin". Biochem.Biophys.Res.Comm. 117, 878-884.

Rothhut, B., Comera, C., Prieur, B., Errasfa, M., Minassian, G. and Russo-Marie, F. (1987) Purification and characterization of a 32-kDa phospholipase A_2 inhibitory protein (lipocortin) from human peripheral blood mononuclear cells. FEBS Lett. 219, 169-175.

Rudge, P., Koetsier, J.C., Mertin, J., Mispelblom Beyer, J.O., Van Walbeek, H.K., Clifford Jones, R., Harrison, J., Robinson, K., Mellein, B., Poole, T., Stokvis, J.C.J.M. and Timonen, P. (1989) Randomised double blind controlled trial of cyclosporin in multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 52, 559-565.

Russo-Marie, F., Paing, M. and Duval, D. (1979) Involvement of glucocorticoid receptors in steroid-induced inhibition of prostaglandin secretion. J.Biol.Chem. 254, 8498-8504.

Russo-Marie, F. and Duval, D. (1982) Dexamethasone-induced inhibition of prostaglandin production does not result from a direct action on phospholipase activities but is mediated through a steroid-inducible factor. Biochim.Biophys.Acta. 712, 177-185.

Sakai, K., Zamvil, S.S., Mitchell, D.J., Hodgkinson, S., Rothbard, J.B. and Steinman, L. (1989) Prevention of experimental encephalomyelitis with peptides blocking T-cell-MHC interaction. Proc.Natl.Acad.Sci.USA 146, 9470-9474.

Sakata, T., Iwagami, S., Tsuruta, Y., Teraoka, H., Hojo, K., Suzuki, S., Sato, K. and Suzuki, R. (1990) The role of lipocortin I in macrophage-mediated immunosuppression in tumor-bearing mice. J.Immunol. 145, 387-396.

Saris, C.J.M., Tack, B.F., Kristensen, T., Glenney, J.R. and Hunter, T. (1986) The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell 46, 201-212.

Schauenstein, K., Fassler, R., Dietrich, H., Schwartz, S., Kromer, G. and Wick, G. (1987) Disturbed immune-endocrine communication in autoimmune disease. Lack of corticosterone response to immune signals in obese strain chickens with spontaneous autoimmune thyroiditis. J.Immunol. 139, 1830-1833.

Schlaepfer, D.D. and Haigler, H.T. (1987) Characterization of Ca2+-dependent phospholipid binding and phosphorylation of lipocortin I. J.Biol.Chem. 262, 6931-6937.

Schlaepfer, D.D. and Haigler, H.T. (1988) In vitro protein kinase C phosphorylation sites of placental lipocortin. Biochemistry 27, 4253-4258.

Schlaepfer, D.D., Fischer, D.A., Brandt, M.E., Bode, H.R., Jones, J.M. and Haigler, H.T. (1992) Identification of a novel annexin in *Hydra vulgaris*. Characterization, cDNA cloning and protein kinase C phosphorylation of annexin XII. J.Biol.Chem. 267, 9529-9539.

Schorlemmer, H.U. and Seiler, F.R. (1991) 15-Deoxyspergualin (15-DOS) for therapy in an animal model of multiple sclerosis (MS): Disease modifying activity on acute and chronic relapsing experimental allergic encephalomyelitis. Agents Actions 34, 156-160.

Sears, E.S., Tindall, R.S.A. and Zarnow, H. (1978) Active multiple sclerosis. Enhanced computerized tomographic imaging of lesions and the effect of corticosteroids. Arch.Neurol. 35, 426-434.

Selmaj, K.W., Farooq, M., Norton, W.T., Raine, C.S. and Brosnan, C.F. (1990) Proliferation of astrocytes *in vitro* in response to cytokines. A primary role for tumor necrosis factor. J.Immunol. 144, 129-135.

Shadle, P.J., Gerke, V. and Weber, K. (1985) Three Ca^{2+}-binding proteins from porcine liver and intestine differ immunologically and physicochemically and are distinct in Ca^{2+} affinities. J.Biol.Chem. 260, 16354-16360.

Shaw, C.M. and Alvord Jr, E.C. (1984) A morphologic comparison of three experimental models of experimental allergic encephalomyelitis with multiple sclerosis. In: Alvord Jr, E.C., Kies, M.W. and Suckling, A.J. (eds.) Experimental allergic encephalomyelitis: a useful model for multiple sclerosis, Alan R.Liss, New York, pp. 61-66.

Sibley, W.A., Kalter, S.S. and Laguna, J.F. (1980) Attempts to transmit multiple sclerosis to newborn and germ-free non-human primates: a ten year interim report. In: Bauer, H.J., Poser, S. and Ritter, G. (eds.) Progress in multiple sclerosis research, Springer-Verlag, New York, pp. 80-88.

Sibley, W.A., Bamford, C.R., Clark, K., Sreith, M.S. and Laguna, J.F. (1991) A prospective study of physical trauma and multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 54, 584-589.

Silva, F.G., Sherrill, K., Spurgeon, S., Sudhof, T.C. and Stone, D.K. (1986) High level expression of the 32.5-kilodalton calelectrin in ductal epithelia as revealed by immunohistochemistry. Differentiation 33, 175-183.

Simmons, R.D., Bernard, C.C.A., Ng, K.T. and Carnegie, P.R. (1981) Hind-limb motor ability in Lewis rats during the onset and recovery phases of experimental autoimmune encephalomyelitis. Brain Res. 215, 103-114.

Simmons, R.D., Bernard, C.C.A., Singer, G. and Carnegie, P.R. (1982) Experimental autoimmune encephalomyelitis. An anatomically-based explanation of clinical progression in rodents. J.Neuroimmunol. 3, 307-318.

Simpson, D.L., Morrison, R., de Vellis, J. and Herschman, H.R. (1982) Epidermal growth factor binding and mitogenic activity on purified populations of cells from the central nervous system. J.Neurosci.Res. 8, 453-462.

Smallwood, M.F., Gurr, S.J., Choudhari, U. and Bowles, D.J. (1990) Characterization of plant annexin gene expression. Biochem.Soc.Trans. 18, 1116.

Smillie, F., Bolton, C., Peers, S.H. and Flower, R.J. (1989a) Distribution of lipocortins I, II and V in tissues of the rat, mouse and guinea pig. Br.J.Pharmacol. 97, 541P.

Smillie, F., Peers, S.H., Elderfield, A-J., Bolton, C. and Flower, R.J. (1989b) Differential regulation by glucocorticoids of intracellular lipocortin I, II and V in rat mixed peritoneal leukocytes. Br.J.Pharmacol. 97, 425P.

Smith, M.E., Somera, F.P. and Eng, L.F. (1983) Immunocytochemical staining for glial fibrillary acidic protein and the metabolism of cytoskeletal proteins in experimental allergic encephalomyelitis. Brain Res. 264, 241-253.

Smith, M.E. and Eng, L.F. (1987) Glial fibrillary acidic protein in chronic relapsing experimental allergic encephalomyelitis in SJL/J mice. J.Neurosci.Res. 18, 203-208.

Smith, M.E. and Eng, L.F. (1988) The development of the gliotic plaque in experimental allergic encephalomyelitis. In: Reier, P.J., Bunge, R.P. and Seil, F.J. (eds.) Current issues in neural regeneration research, Alan R.Liss, New York, pp. 291-300.

Smith, S.F., Goulding, N.J., Tetley, T.D., Godolphin, J.L., Guz, A. and Flower, R.J. (1989a) A possible anti-inflammatory mechanism for glucocorticoids in the human lung. Clin.Sci. 76 (suppl. 21), 20P.

Smith, T., Flower, R.J. and Buckingham, J.C. (1989b) The presence of lipocortin-like proteins in the brain and pituitary gland of the rat. J.Endocrinol. 123, 46P.

Smith, S.F., Goulding, N.J., Godolphin, J.L., Tetley, T.D., Roberts, C.M., Guz, A. and Flower, R.J. (1990a) An assay for the assessment of lipocortin 1 levels in human lung lavage fluid. J.Immunol.Methods 131, 119-125.

Smith, S.F., Tetley, T.D., Guz, A. and Flower, R.J. (1990b) Detection of lipocortin 1 in human lung lavage fluid: Lipocortin degradation as a possible proteolytic mechanism in the control of inflammation. Environ.Health Perspect. 85, 135-144.

Smith, T., Flower, R.J. and Buckingham, J.C. (1990c) Effects of dexamethasone treatment on the lipocortin I content of specific brain nuclei in the rat. J.Endocrinol. 127 (suppl.), P49.

Smith, V.L. and Dedman, J.R. (1986) An immunological comparison of several novel calcium-binding proteins. J.Biol.Chem. 261, 15815-15818.

Snedecor, G.W. and Cochran, W.G. (1967) Statistical methods, Iowa State University Press, Iowa, Ed. 6th.

Snyder, D.H., Valsamis, M.P., Stone, S.H. and Raine, C.S. (1975) Progressive demyelination and reparative phenomena in chronic experimental allergic encephalomyelitis. J.Neuropathol.Exp.Neurol. 34, 209-221.

Solito, E. and Parente, L. (1989) Modulation of phospholipase A_2 activity in human fibroblasts. Br.J.Pharmacol. 96, 656-660.

Solito, E., Raugei, G., Melli, M. and Parente, L. (1990) Effect of dexamethasone and phorbol myristate acetate on lipocortin 1, 2 and 5 mRNA and protein synthesis. In: Samuelsson, B., Ramwell, P.W., Paoletti, R., Folco, G. and Gramstrom, E. (eds.) Advances in prostaglandin, thromboxane and leukotriene research, vol. 20, Raven Press, New York, pp. 291-294.

Solito, E., Raugei, G., Melli, M. and Parente, L. (1991) Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated but not undifferentiated U937 cells. FEBS Lett. 291, 238-244.

Steiner, I., Brenner, T., Mizrachi-Kol, R. and Abramsky, O. (1991) Development of experimental allergic encephalomyelitis during steroid administration. Outcome of neurological immune-mediated disorders under immunosuppressive therapy. Isr.J.Med.Sci. 27, 365-368.

Steinman, L., Rosenbaum, J., Sriram, S. and McDevitt, H.O. (1981) *In vivo* effects of antibodies to immune response gene products: Prevention of experimental allergic encephalomyelitis. Proc.Natl.Acad.Sci.USA 78, 7111-7114.

Stevens, T.R.J., Drasdo, A.L., Peers, S.H., Hall, N.D. and Flower, R.J. (1988) Stimulus-specific inhibition of human neutrophil H_2O_2 production by human recombinant lipocortin 1. Br.J.Pharmacol. 93, 139P.

Stott, D.I. (1989) Immunoblotting and dot blotting. J.Immunol.Methods 119, 153-187.

Strijbos, P.J.L.M., Tilders, F.J.H., Carey, F., Forder, R. and Rothwell, N.J. (1991) Localization of immunoreactive lipocortin-1 in the brain and pituitary gland of the rat. Effects of adrenalectomy, dexamethasone and colchicine treatment. Brain Res. 553, 249-260.

Stuart, G. and Krikorian, K.S. (1928) The neuro-paralytic accidents of anti-rabies treatment. Ann.Trop.Med.Parasitol. 22, 327-377.

Sudhof, T.C., Slaughter, C.A., Leznicki, I., Barjon, P. and Reynolds, G.A. (1988) Human 67-kDa calelectrin contains a duplication of four repeats found in 35 kDa lipocortins. Proc.Natl.Acad.Sci.USA 85, 664-668.

Swanson, J.W. (1989) Multiple sclerosis: Update in diagnosis and review of prognostic factors. Mayo Clin.Proc. 64, 577-586.

Tait, J.F., Sakata, M., McMullen, B.A., Miao, C.H., Funakoshi, T., Hendrikson, L.E. and Fujikawa, K. (1988) Placental anticoagulant proteins: isolation and comparative characterization of four members of the lipocortin family. Biochemistry 17, 6268-6276.

Teasdale, G.M., Smith, P.A., Wilkinson, R., Latner, A.L. and Miller, H. (1967) Endocrine activity in multiple sclerosis. Lancet 1, 64-68.

Thompson, A.J., Kennard, C., Swash, M., Summers, B., Yuill, G.M., Shepherd, D.I., Roche, S., Perkin, G.D., Loizou, L.A., Ferner, R., Hughes, R.A.C., Thompson, M. and Hand, J. (1989) Relative efficacy of intravenous methylprednisolone and ACTH in the treatment of acute relapse in MS. Neurology 39, 969-971.

Thompson, A.J., Kermode, A.G., MacManus, D.G., Kendall.B.E., Kingsley, D.P.E., Moseley, I.F. and McDonald, W.I. (1990) Patterns of disease activity in multiple sclerosis: clinical and magnetic resonance imaging study. Br.Med.J. 300, 631-634.

Tindall, R. (1988) A closer look at plasmapheresis in multiple sclerosis: the cons. Neurology 38 (suppl. 2), 53-56.

Tourtellotte, W.W. and Haerer, A.F. (1965) Use of an oral corticosteroid in the treatment of multiple sclerosis. Arch.Neurol. 12, 536-545.

Tourtellotte, W.W., Baumhefner, R.W., Potvin, A.R., Ma, B.I., Potvin, J.H., Mendez, M. and Syndulko, K. (1980) Multiple sclerosis de novo CNS IgG synthesis: effect of ACTH and corticosteroids. Neurology 30, 1155-1162.

Tourtellotte, W.W., Baumhefner, R.W., Syndulko, K., Shapshak, P., Osborne, M., Rubinshtein, G., Newton, L., Ellison, G., Myers, L., Rosario, I., Thomsen, R., Sloan, R. and Engelmann, S. (1988) The long march of the cerebrospinal fluid profile indicative of clinical definate multiple sclerosis; and still marching. J.Neuroimmunol. 20, 217-227.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc.Natl.Acad.Sci.USA 76, 4350-4354.

Towle, C.A. and Treadwell, B.V. (1992) Identification of a novel mammalian annexin. cDNA cloning, sequence analysis, and ubiquitous expression of the annexin XI gene. J.Biol.Chem. 267, 5416-5423.

Traugott, U., Shevach, E., Chiba, J., Stone, S.H. and Raine, C.S. (1982) Acute experimental autoimmune encephalomyelitis: T- and B-cell distribution within the target organ. Cell.Immunol. 70, 345-356.

Traugott, U., Reinherz, E.L. and Raine, C.S. (1983) Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. J.Neuroimmunol. 4, 201-221.

Traugott, U., Scheinberg, L.C. and Raine, C.S. (1985) On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. J.Neuroimmunol. 8, 1-14.

Traugott, U. (1989) Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. Cell.Immunol. 119, 114-129.

Troiano, R., Hafstein, M., Ruderman, M., Dowling, P. and Cook, S. (1984) Effect of high-dose intravenous steroid administration on contrast-enhancing computed tomographic scan lesions in multiple sclerosis. Ann.Neurol. 15, 257-263.

Troiano, R., Cook, S.D. and Dowling, P.C. (1987) Steroid therapy in multiple sclerosis: point of view. Arch.Neurol. 44, 803-807.

Troiano, R., Cook, S.D. and Dowling, P.C. (1989) Is steroid therapy in multiple sclerosis superior to corticotrophin therapy. Arch.Neurol. 46, 362.

Trotter, J. and Smith, M.E. (1984) Macrophage-mediated demyelination: The role of phospholipases and antibody. In: Alvord Jnr., E.C., Kies, M.W. and Suckling, A.J. (eds.) Experimental allergic encephalomyelitis: a useful model for multiple sclerosis, Alan R. Liss, New York, pp. 55-60.

Tung, J-S. and Knight, C.A. (1972) Relative importance of some factors affecting electrophoretic migration of proteins in sodium dodecyl sulphate-polyacrylamide gels. Anal.Biochem. 48, 153-163.

Uede, T., Hirata, F., Hirashima, M. and Ishizaka, K. (1983) Modulation of the biologic activities of IgE-binding factors. 1. Identification of glycosylation-inhibiting factor as a fragment of lipomodulin. J.Immunol. 130, 878-884.

Uitdehaag, B.M.J., Polman, C.H., de Groot, C.J.A., Huitinga, I. and Dijkstra, C.D. (1991) Failure of sulphasalazine to influence experimental autoimmune encephalomyelitis. Acta Neurol.Scand. 84, 173-174.

Valentine-Braun, K.A., Hollenberg, M.D., Fraser, E. and Northup, J.K. (1987) Isolation of a major human placental substrate for the epidermal growth factor (urogastrone) receptor kinase: immunological cross-reactivity with transducin and sequence homology with lipocortin. Arch.Biochem.Biophys. 259, 262-282.

van Binsbergen, J., Slotboom, A.J., Aarsman, A.J. and de Haas, G.H. (1989) Synthetic peptide from lipocortin I has no phospholipase A_2 inhibitory activity. FEBS Lett. 247, 293-297.

Vandenbark, A.A., Hashim, G. and Offner, H. (1989) Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. Nature 341, 541-544.

Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. Nature 231, 232-235.

Varticovski, L., Chahwala, S.B., Whitman, M., Cantley, L., Schindler, D., Chow, E.P., Sinclair, L.K. and Pepinsky, R.B. (1988) Location of sites in human lipocortin I that are phosphorylated by protein tyrosine kinases and protein kinases A and C. Biochemistry 27, 3628-3690.

Vela, L., Garcia Merino, A., Fernandez-Gallardo, S., Sanchez Crespo, M., Lopez Lozano, J.J. and Saus, C. (1991) Platelet-activating factor antagonists do not protect against the development of experimental autoimmune encephalomyelitis. J.Neuroimmunol. 33, 81-86.

Violette, S.M., King, I., Browning, J.L., Pepinsky, R.B., Wallner, B.P. and Sartorelli, A.C. (1990) Role of lipocortin I in the glucocorticoid induction of the terminal differentiation of a human squamous carcinoma. J.Cell.Physiol. 142, 70-77.

Vishwanath, B.S., Frey, F.J., Bradbury, M., Dallman, M.F. and Frey, B.M. (1992) Adrenalectomy decreases lipocortin-I messenger ribonucleic acid and tissue protein content in rats. Endocrinology 130, 585-591.

Vogel, F.S. (1950) A lipolytic enzyme in reactive histiocytes of guinea pigs with experimental encephalomyelitis. J.Exp.Med. 93, 305-315.

Voorthuis, J.A.C., Uitdehaag, B.M.J., De Groot, C.J.A., Goede, P.H., van der Meide, P.H. and Dijkstra, C.D. (1990) Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. Clin.Exp.Immunol. 81, 183-188.

Wajgt, A., Gorny, M. and Jenek, R. (1983) The influence of high-dose prednisone medication on autoantibody specific activity and on circulating immune complex level in cerebrospinal fluid of multiple sclerosis patients. Acta Neurol.Scand. 68, 378-385.

Waksman, B.H. and Reynolds, W.E. (1984) Multiple sclerosis as a disease of immune regulation. Proc.Soc.Exp.Biol.Med. 175, 282-294.

Walker, J. (1982) Isolation from cholinergic synapses of a protein that binds to membranes in a calcium-dependent manner. J.Neurochem. 39, 815-823.

Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Chow, E.P., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature 320, 77-81.

Walsh, M.J., Tourtellotte, W.W., Potvin, A.R. and Potvin, J.H. (1983) The cerebrospinal fluid in multiple sclerosis. In: Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W. (eds.) Multiple sclerosis, Chapman and Hall, London, pp. 275-358.

Warren, K.G., Catz, I. and Verona, M.J. (1986) Effects of methylprednisolone on CSF IgG parameters, myelin basic protein and anti-myelin basic protein in multiple sclerosis exacerbations. Can.J.Neurol.Sci. 13, 25-30.

Warren, S., Greenhill, S. and Warren, K.G. (1982) Emotional stress and the development of multiple sclerosis: case control evidence of a relationship. J.Chronic Dis. 35, 821-831.

Weller, R.O. (1985) Pathology of multiple sclerosis. In: Mathews, W.B. (ed.) McAlpine's multiple sclerosis, Churchill Livingstone, London, pp. 301-343.

Werb, Z., Foley, R. and Munck, A. (1978) Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages. J.Exp.Med. 147, 1684-1694.

Wisniewski, H.M. (1977) Immunopathology of demyelination in autoimmune diseases and virus infections. Br.Med.Bull. 33, 54-59.

Wisniewski, H.M., Lassmann, H., Brosnan, C.F., Mehta, P.D., Lidsky, A.A. and Madrid, R.E. (1982) Multiple sclerosis: Immunological and experimental aspects. In: Mathews, W.B. and Glaser, C.H. (eds.) Recent advances in neurology, Churchill Livingstone, Edinburgh, pp. 95-124.

Woelk, H., Kanig, K. and Peiler-Ichikawa, K. (1974) Phospholipid metabolism in experimental allergic encephalomyelitis: activity of mitochondrial phospholipase A_2 of rat brain towards specifically labelled 1,2-diacyl-1-alk-1'-enyl-2-acyl and 1-alkyl-2-acyl-SN-glycero-3-phosphorylcholine. J.Neurochem. 23, 745-750.

Woelk, H., Jakumeit-Morgott, U. and Schenck, K. (1976) Phospholipid metabolism in subacute sclerosing panencephalitis: activity of brain phospholipase A_2 towards specifically labelled 1,2-diacyl-1-alk-1'-enyl-2-acyl and 1-alkyl-2-acyl-SN-glycero-3-phosphorylcholine. J.Neurochem. 26, 275-279.

Wong, W.T., Frost, S.C. and Nick, H.S. (1991) Protein-synthesis-dependent induction of annexin I by glucocorticoid. Biochem.J. 275, 313-319.

Woolgar, J.A., Boustead, C.M. and Walker, J.H. (1990) Characterization of annexins in mammalian brain. J.Neurochem. 54, 62-71.

Zamvil, S.S. and Steinman, L. (1990) The T lymphocyte in experimental allergic encephalomyelitis. Annu.Rev.Immunol. 8, 579-621.

Zokas, L. and Glenney, J.R. (1987) The calpactin light chain is highly linked to the cytoskeletal form of calpactin I - studies using monoclonal antibodies to calpactin subunits. J.Cell Biol. 105, 2111-2121.

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The detection of lipocortins 1, 2 and 5 in central nervous system tissues from Lewis rats with acute experimental allergic encephalomyelitis

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Summary

The calcium and phospholipid-binding proteins lipocortins 1, 2 and 5 have been detected, by electrotransfer and immunoblotting techniques, in central nervous system (CNS) tissues of control Lewis rats and animals with experimental allergic encephalomyelitis (EAE). The cerebellum and spinal cord content of lipocortins 2 and 5 remained unchanged throughout the development of EAE but the amounts of the steroid-inducible protein lipocortin 1 increased in samples from pre-diseased and adjuvant-treated rats and were further enhanced in tissues from clinically sick and convalescent animals. The significance of these findings is discussed in conjunction with the ongoing changes in pathology which occur during the progression of EAE.

Introduction

Experimental allergic encephalomyelitis (EAE) has been extensively studied and widely used as an animal model of the human disabling disease multiple sclerosis (MS) (Paterson, 1976; Raine, 1984). Depending upon the species used and the method of induction, EAE can be characterised clinically by either acute paralytic signs or chronic relapsing-remitting episodes of limb dysfunction (Lassmann, 1983). One feature common to models of the disease is the infiltration of the central nervous system (CNS) by inflammatory-type cells consisting mainly of lymphocytes and macrophages. Many studies have shown that factors derived from cells of the lymphocyte-macrophage series can initiate and perpetuate the inflammatory response (Humes et al., 1977; Nathan et al., 1982; Geczy, 1984; Davies, 1985). In EAE, the brains and spinal cords of diseased animals have been shown to contain high levels of eicosanoids and lysosomal enzymes both of which may contribute to the increase in vascular permeability, oedema

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and demyelination observed during the course of the disease (Smith et al., 1974; Smith, 1979; Bolton et al., 1984a, b).

One important inflammatory cell product implicated in myelin breakdown and a pre-requisite for mediator production is the enzyme phospholipase A_2 (PLA_2) (Woelk and Kanig, 1974; Fu et al., 1980; Trotter and Smith, 1986). Over the past decade much evidence has accumulated to show that the effects of PLA_2 may be controlled by the synthesis and release of glucocorticoid-inducible proteins. One such protein commanding particular attention is lipocortin 1, for which the structure has been elucidated, sequenced and cloned (Flower and Blackwell, 1979; Blackwell et al., 1980, 1982; Pepinsky et al., 1986; Wallner et al., 1986). Early reports on the distribution of lipocortin-like proteins revealed the brain and macrophage as prominent sources (Flower, 1988) and recent studies have shown an increased presence of lipocortin 1 in diseased human CNS tissues to be associated with invading and resident macrophages (Johnson et al., 1989a, b). In addition, in vivo treatment with dexamethasone has been shown to raise basal levels of lipocortin 1 in rat brain by several fold and induce the synthesis and release of the protein from peritoneal and alveolar macrophages (Blackwell et al., 1982; Flower, 1984). Thus, it is evident that the potential exists, within the target tissues of EAE-diseased animals, for the control of PLA_2-mediated inflammation via the limiting actions of lipocortin 1, the levels of which may be influenced by exogenous or endogenous steroids. In this study CNS tissues from control rats and animals in various stages of EAE have been assessed for the presence of lipocortin 1 and the structurally related lipocortins 2 and 5. Possible sources of the lipocortins are considered and the relevance of the findings in relation to steroid function is discussed.

Materials and methods

Animals

Male Lewis rats (Bantin and Kingman, Hull, U.K.) weighing 200–250 g were used. The animals, housed six per cage, were maintained on Labsure CRM rat diet and water ad libitum.

Induction of EAE

Two weeks were allowed for the rats to adapt to their new environment before inoculation with encephalitogen. Each treatment group contained six animals which were injected in each hind footpad with 0.1 ml of an emulsion consisting of equal parts of guinea pig spinal cord, sterile phosphate-buffered saline (PBS) and Freund's incomplete adjuvant (FIA) containing 10 mg/ml *Mycobacterium tuberculosis* $H_{37}Ra$ (Difco, Surrey, U.K.). An equal volume of PBS was substituted for spinal tissue in control rats receiving Freund's complete adjuvant (FCA) (equivalent to FIA plus mycobacteria).

Clinical evaluation of EAE

Animals were examined for clinical signs of disease beginning 5 days post-inoculation (p.i.) and continuing for the duration of the experiment. Three phases in the development of EAE were chosen to determine the lipocortin content of CNS tissues. Phase 1: 6–7 days p.i., and coinciding with the presence, in target tissues, of neuroreactive T cells (Traugott et al., 1981, 1982). Phase 2: at the height of disease, 13–15 days p.i., and characterised by complete hind limb paralysis. Phase 3: immediately after the total loss of neurological signs of EAE, 18–20 days p.i. The CNS tissues of rats receiving CFA alone were removed 14 days p.i. corresponding with the time of maximum disease intensity in EAE-inoculated animals.

Preparation of CNS tissues for immunoelectrophoretic and histological techniques

Rats were asphyxiated in carbon dioxide, exsanguinated and the cerebellum and first 3 cm of spinal cord dissected, washed in ice-cold PBS to remove any excess blood and carefully blotted dry. CNS tissues were longitudinally bisected and the left side of each sample was stored in 10% formal saline for subsequent histological processing (see below) while the remaining half was frozen at $-20\,^{\circ}C$ until required.

Individual thawed tissues were cut up in 0.5 ml of chilled medium, pH 7.6, containing 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulphonic acid, sodium salt (Hepes) (Sigma), 5 mM ethylenediaminetetraacetic acid (disodium salt dihydrate) (EDTA) (Sigma), 200 μM phenylmethyl-

sulphonyl fluoride (PMSF) (Sigma) and 100 mM sodium chloride (Sigma). In order to visualise lipocortin in CNS tissues, an homogenate of each sample was made by equal and repeated aspiration, at 4° C through a 19 and 21 gauge disposable needle, followed by three cycles of freeze-thawing. Supernatants were prepared by centrifugation of disrupted tissues at 30,000 × *g* for 30 min at 4° C. The protein content of each sample was determined by the method of Bradford (1976), adjusted to 2 mg/ml and aliquots were mixed, and transferred to a boiling water bath for 5 min, after first adding an equal volume of buffer containing 0.1 M Tris (hydroxymethyl)aminomethane/(*N*,*N*-bis[2-hydroxyethyl]glycine) (Tris/Bicine) (Sigma), 2% sodium dodecyl sulphate (SDS) (BDH), 5% 2-mercaptoethanol (Fisons), 10% sucrose (BDH) and 0.05% bromophenol blue (BDH). Cooled samples were stored at −20° C until required for polyacrylamide gel electrophoresis (PAGE).

PAGE and electroblotting of CNS samples

PAGE was performed essentially as described by Laemmli (1970). Briefly, equal volumes from individual samples were pooled and 20 μl aliquots, containing 20 μg of protein, from each treatment were applied to SDS/polyacrylamide gels and constituent proteins were separated by electrophoresis using a 'Mighty Small II' vertical slab gel unit (Hoefer Scientific Instruments, U.S.A.). Gels were either stained with 0.2% PAGE blue 83 (BDH) to visualise individual bands or subjected to electroblotting, using a Transphor electrophoresis unit (Hoefer Scientific Instruments, U.S.A.) to facilitate the transfer of proteins onto nitrocellulose sheets.

Immunoblotting of CNS proteins with antibodies against lipocortins 1, 2 and 5

Following transfer, any unoccupied protein binding sites on the nitrocellulose membranes were blocked by agitation in PBS containing 3% milk protein plus 0.1% Tween 20. CNS proteins were then probed overnight with polyclonal rabbit antisera directed against either human recombinant lipocortin 1 (code no. 842), native human lipocortin 2 (code no. 774) or native rat lipocortin 5 (code no. 890) (generously supplied by Dr. J.L. Browning, Biogen Research Corporation), diluted 1 in 5000 with PBS/0.1% Tween 20. Cross-reactivity data for the lipocortins are as stated by Pepinsky et al. (1988). Each lipocortin was detected by incubating respective blots with a 1 in 1000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) followed by colour development in a solution containing 0.05% diaminobenzidine hydrochloride (DAB) (Sigma) and 0.02% hydrogen peroxide (BDH).

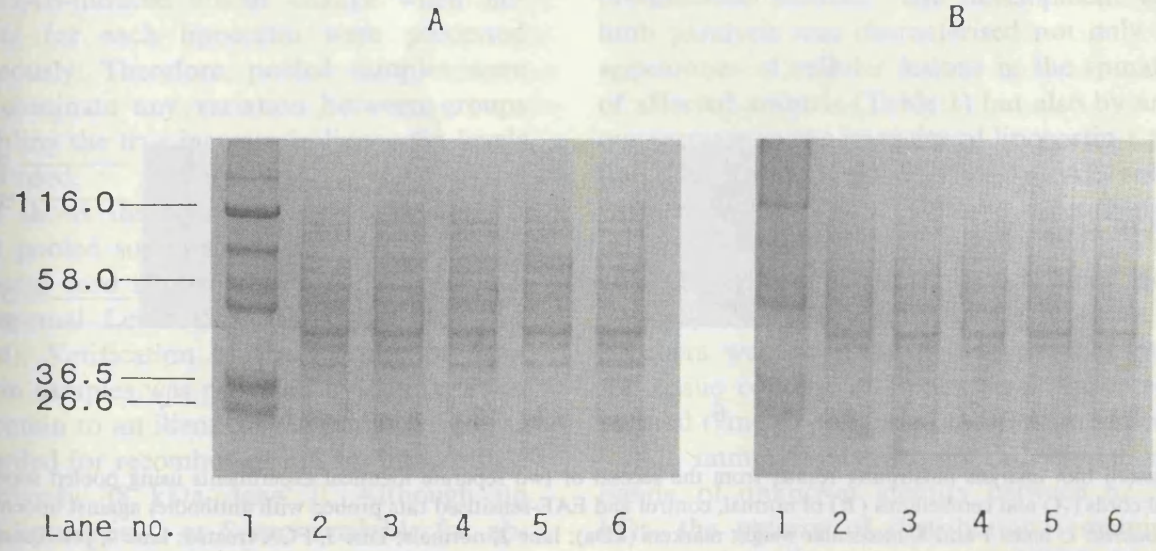

Fig. 1. SDS-PAGE of pooled spinal cord (*A*) and cerebellum (*B*) supernatants from normal Lewis rats (lane 2), controls receiving FCA (lane 3) and animals immunised with adjuvant plus guinea pig spinal cord before the onset of EAE (lane 4), at the time of paralysis (lane 5) and during recovery from disease (lane 6). Molecular weight markers (lane 1) are expressed in kDa.

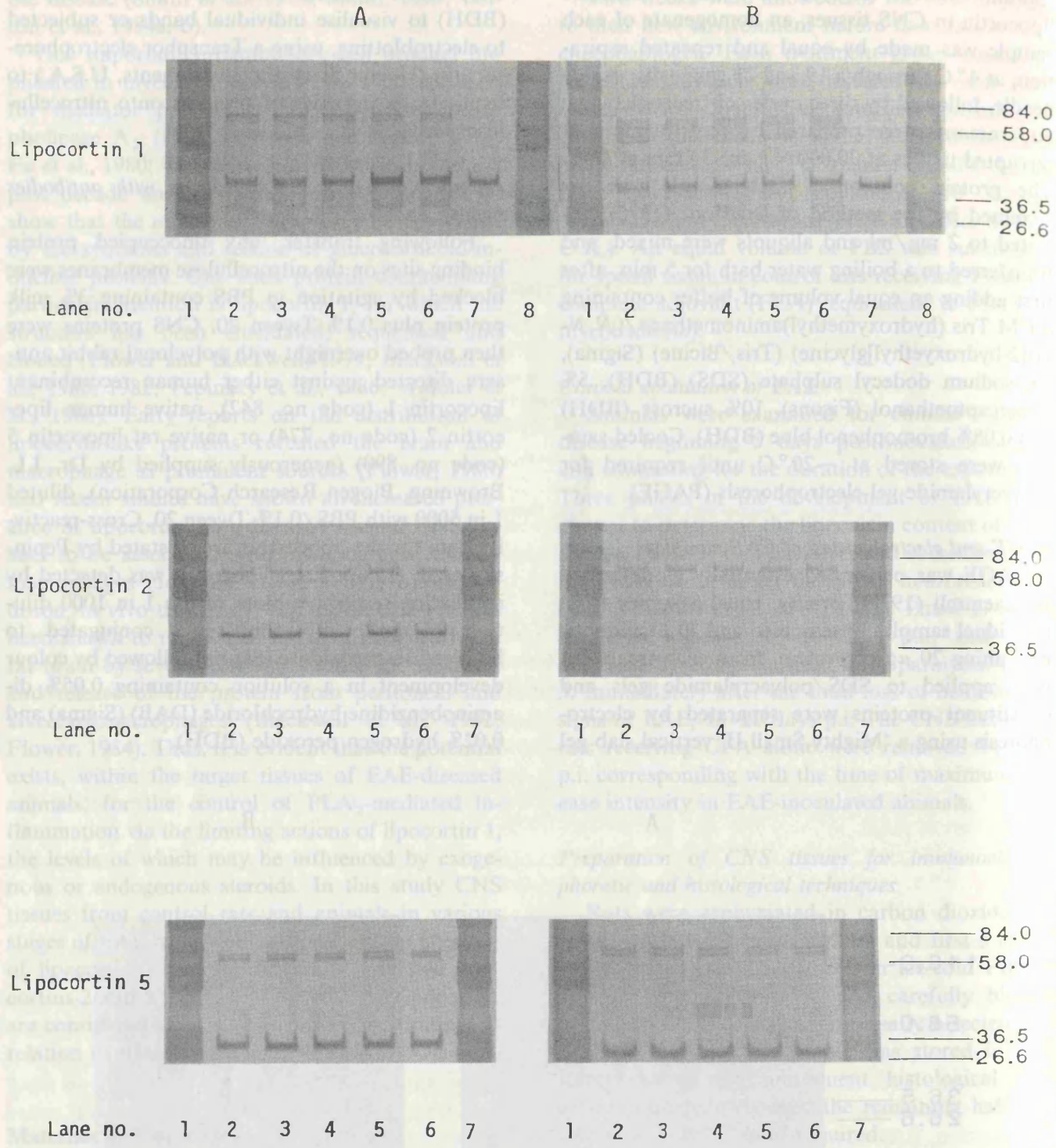

Fig. 2. Western blot analysis illustrating results from the second of two separate identical experiments using pooled supernatants from spinal cords (*A*) and cerebellums (*B*) of normal, control and EAE-sensitised rats probed with antibodies against lipocortins 1, 2 and 5. Lipocortin 1: lanes 1 and 8, molecular weight markers (kDa); lane 2, normals; lane 3, FCA-treated; lane 4, pre-diseased; lane 5, clinically diseased; lane 6, recovered; lane 7, recombinant human lipocortin 1 (25 ng/immunoblot). Lipocortins 2 and 5: lanes 1 and 7, molecular weight markers (kDa); lane 2, normals; lane 3, FCA-treated; lane 4, pre-diseased; lane 5, clinically diseased; lane 6, recovered.

Estimation of inflammatory infiltrates in CNS tissues

Longitudinal-horizontal sections, at one standard depth, were taken from the spinal cords and cerebellums of control and EAE-inoculated animals. After staining with haematoxylin/eosin the number of perivascular infiltrates in each section was determined by light microscopy.

Results

SDS-PAGE of pooled CNS supernatants from normal, control and EAE-inoculated rats

Fig. 1*A* and *B* shows that SDS-PAGE separates pooled spinal cord and cerebellum supernatants into constituent proteins enabling their transfer onto nitrocellulose membranes for subsequent immunoblotting with relevant antibodies. Visual examination of the stained protein bands did not reveal any constituent differences between treatments.

Detection of lipocortins 1, 2 and 5 following electrotransfer and immunoblotting of CNS proteins

Preliminary experiments revealed no single sample within any of the groups contained excessively high levels of lipocortin compared to others in the same treatment (data not shown). Initial studies also showed slight variation in the intensity of DAB-induced colour change when immunoblots for each lipocortin were processed simultaneously. Therefore, pooled samples were used to eliminate any variation between groups thus enabling the true increase in lipocortin levels to be recorded.

Fig. 2 shows the presence of lipocortins 1, 2 and 5 in pooled supernatants prepared from the upper spinal cord segments (*A*) and cerebellums (*B*) of normal Lewis rats (lane 2 in each immunoblot). Verification of the identity of lipocortin 1 in samples was provided by co-migration of the protein to an identical molecular weight as that recorded for recombinant human lipocortin 1 (approximately 38 kDa, lane 7). Although no standard lipocortin 2 or 5 was available for absolute authentication of these proteins in samples the principal bands observed following incubation with anti-lipocortin 2 and 5 antibodies had similar molecular weights (37 kDa and 35 kDa respectively) to those reported by Pepinsky et al. (1988).

TABLE 1

THE OCCURRENCE OF PERIVASCULAR INFILTRATES IN CNS TISSUES DURING THE COURSE OF ACUTE EAE

Treatment	Number of lesions in sections cut at one standard depth/tissue (±SD)	
	Spinal cord	Cerebellum
Normals	ND	ND
FCA-treated	0	0
Pre-diseased	0	0
Clinically diseased	54±13	2±2
Clinically recovered	29±10	4±3

CNS tissues from animals receiving FCA appeared to contain more lipocortin 1 compared to normals but the amounts of lipocortin 2 and 5 in controls did not vary (lanes 3). Indeed, no appreciable differences occurrred in the lipocortin 2 or 5 content of CNS samples from any of the treatments analysed. Samples derived from spinal cords and cerebellums of rats immunised 6–7 days previously with adjuvant plus antigen showed a comparable degree of staining for lipocortin 1 (lanes 4) to that seen in tissues from animals inoculated with FCA alone. Also the intensity of staining in cerebellum supernatants from rats with paralytic EAE (lane 5) was equivalent to that observed in pre-diseased animals. The development of hind limb paralysis was characterised not only by the appearance of cellular lesions in the spinal cords of affected animals (Table 1) but also by an obvious increase in the quantity of lipocortin 1 present (lane 5). Recovery from clinical EAE coincided with a reduction in the number of inflammatory infiltrates in spinal tissue together with a noticeable diminution of lipocortin 1 band intensity (lane 6). A slight increase in cerebellum lesion numbers was observed in convalescent rats and the tissue content of lipocortin 1 appeared augmented (lane 6) compared to other treatments.

All immunoblots showed additional distinct bands, of unknown identity between 62 and 70 kDa, the pattern of distribution remaining unchanged throughout the course of EAE. It is possible that these higher molecular weight bands are multimers of lipocortin 1 and similar to those

described by Ando et al. (1989) and Pepinsky et al. (1989). In addition, low molecular weight breakdown products of the lipocortins were minimal and, in the case of those present after probing with anti-lipocortin 1 antibody (approximately 33 kDa), appeared proportional to the intensity of the parent band.

Discussion

Results presented in this study describe the presence of lipocortins 1, 2 and 5 in the CNS tissues of normal, control and EAE-inoculated Lewis rats. The work also reveals, for the first time, that defined areas of the CNS removed from animals receiving CFA alone or together with guinea pig spinal cord contain increased quantities of the glucocorticoid-inducible protein lipocortin 1. Furthermore, the greatest amounts of lipocortin 1 were detected in tissues infiltrated by inflammatory cells and removed from clinically diseased or convalescent rats.

The lipocortins are a family of calcium and phospholipid-binding proteins which are capable of inhibiting the actions of PLA_2 in vitro and producing a systemic anti-inflammatory effect. Recent data has established the lipocortins as members of a wider group of proteins, collectively termed chromobindins, which share both functional and sequence homology and act as substrates for protein tyrosine kinase (Hollenberg et al., 1988). Although no information exists on the regulation of endogenous PLA_2 activity by the lipocortins it may be that down-regulation of the actions of phospholipase by these proteins could assist in limiting the course of inflammation in vivo.

Characterisation of the proteins has revealed that lipocortins 1, 2 and 5 are structurally related and have approximate masses of 38 kDA, 38 kDa and 35 kDa respectively (Pepinsky et al., 1988) which compare to the molecular weights of the proteins detected in the current investigation. Knowledge of the functional properties of lipocortins 1, 2 and 5 is limited to their in vitro biochemical activity and prominence as intrinsic cellular proteins (Comera et al., 1989). Interestingly the levels of lipocortins 2 and 5 did not change in CNS tissues with the progression of EAE suggesting that the proteins are not dramatically affected by immunologically directed inflammatory cell invasion of target areas. In contrast, information on lipocortin 1 is more extensive, encompassing its distribution, in vitro and in vivo activities and the relationship between the protein and steroid actions. In vitro, lipocortin 1 has been shown to inhibit eicosanoid and lysoplatelet activating factor release and in vivo to suppress models of pleurisy and paw oedema (Blackwell et al., 1982; Parente et al., 1984; Parente and Flower, 1985; Miele et al., 1988; Errasfa and Russo-Marie, 1989). Studies by Cirino and colleagues using human recombinant lipocortin 1 have shown prevention of eicosanoid release from lung and arteriole tissue and, when administered locally, inhibition of irritant-induced paw swelling (Cirino and Flower, 1987; Cirino et al., 1987, 1989).

To date the unequivocal demonstration of lipocortin induction by steroids in every test system used remains elusive but many studies have shown increased levels of the protein in tissues, cells and lavage fluid following in vitro or in vivo treatment with the glucocorticoids (Hirata et al., 1980; Flower, 1984; Errasfa et al., 1985; Lundgren et al., 1988; Goulding et al., 1989; Piltch et al., 1989; Smith et al., 1989). The current study has shown an intensified presence of lipocortin 1 in cerebellums and spinal cords from inoculated animals but without the involvement of exogenous steroids. Control tissue from rats receiving adjuvant alone, for 2 weeks, contained more lipocortin 1 than normal samples and similar amounts to those detected in pre-diseased animals (Fig. 2*A* and *B*, lanes 2, 3 and 4). In order to offer an explanation for the changes in lipocortin 1 levels observed in these inoculated rats the effects of in vivo exposure to antigen(s) and the clinical status of the animals at the time of sampling should be considered. Work by Besedovsky et al. (1975) and Schauenstein et al. (1987) demonstrated that during the first week following inoculation with an antigen a significant rise in circulating glucocorticoids can be detected. Also, a recent and particularly relevant study by Mackenzie et al. (1989) showed that serum corticosterone in Lewis rats was elevated 3–7 days after inoculation for

EAE. In addition, these workers found that animals immunised with FCA developed significantly raised glucocorticoid levels approximately 2 weeks p.i. It should be noted that the injection of FCA into Lewis and other strains of rats is known to cause experimental arthritis (Pearson, 1956; van Eden et al., 1985) with resulting stress due to chronic inflammation, physiological dysfunction and pain (De Castro Coster et al., 1981; Colpaert et al., 1982). Increased circulating levels of glucocorticoids either as a result of antigenic stimulation or stress-induced changes may act similarly to exogenous steroids and facilitate the induction of lipocortin 1 synthesis. Consequently, such a response, perhaps together with additional factors such as circulating cytokines, may account for the augmentation of lipocortin 1 in cerebellum and spinal cords from animals receiving adjuvant in the presence or absence of encephalitogen.

The development of stress as a consequence of EAE and the concomitant release of immunosuppressive quantities of corticosterone was thought to occur during the development of the disease and to be responsible for the characteristic loss of neurological signs (Levine et al., 1962; Levine and Strebel, 1969). Subsequent studies by MacPhee et al. (1989) and MacKenzie et al. (1989) have confirmed a significant increase in circulating corticosterone during the course of EAE and work by the authors (Bolton and Flower, 1989) using the anti-glucocorticoid RU38486 to antagonise the beneficial effects of endogenous steroids has provided additional information on their ability to 'self cure' in rat models of EAE. The stress-induced release of glucocorticoids into the systemic circulation of EAE-diseased rats may have similar effects to those generated in adjuvant-sensitised animals and thus cause an induction of lipocortin 1 in the CNS and other tissues. The potential for additional amounts of lipocortin 1 to be present in cerebellum and spinal cords of sick and convalescent rats could be provided by a rich source of the protein in the form of invasive macrophages known to be present in perivascular lesions such as those quantitated in the present study. Therefore, it is possible that inflammatory cell-derived lipocortin 1 in combination with an indirect induction of the protein, via endogenous steroids, accounts for the increased intensity of staining seen when examining CNS samples from animals during and after clinical signs of EAE (Fig. 2*A* and *B*, lanes 5 and 6).

To summarise, evidence has been provided to show the existence of three forms of lipocortin in the CNS tissues from control rats and animals with the acute form of EAE. Although the amount of lipocortins 2 and 5 present in samples did not change with the development of disease, the quantity of the steroid-inducible protein lipocortin 1 increased in inoculated rats and was further enhanced in tissues removed from clinically diseased or recovered animals. Studies are in progress to identify the source of CNS lipocortins and determine the effects of endogenous and exogenous steroids on the induction of lipocortin 1. It is hoped that a clearer understanding of the mechanisms by which steroids exert their effects in EAE will be achieved together with an improvement in our knowledge of steroid action and therapy in human demyelinating diseases such as MS.

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References

Ando, Y., Imamura, S., Owada, M.K., Kakunaga, T. and Kannagi, R. (1989) Cross-linking of lipocortin 1 and enhancement of its Ca^{2+} sensitivity by tissue transglutaminase. Biochem. Biophys. Res. Commun. 163, 944–951.

Besedovsky, H., Sorkin, E., Keller, M. and Muller, J. (1975) Changes in blood hormone levels during the immune response. Proc. Soc. Exp. Biol. Med. 150, 466–470.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J., Parente, L. and Persico, P. (1980) Macrocortin: a polypeptide causing the anti-phospholipase effects of glucocorticoids. Nature 287, 147–149.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J.,. Langham, C.S.J., Parente, L., Persico, P., Russell-Smith, N.C. and Stone, D. (1982) Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. Br. J. Pharmacol. 76, 185–194.

Bolton, C. and Flower, R.J. (1989) The effects of the anti-glucocorticoid RU38486 on steroid-mediated suppression of experimental allergic encephalomyelitis (EAE) in the Lewis rat. Life Sci. 45, 97–104.

Bolton, C., Gordon, D. and Turk, J.L. (1984a) A longitudinal study of the prostaglandin content of central nervous system tissues from guinea pigs with acute experimental allergic encephalomyelitis (EAE). Int. J. Immunopharm. 6, 155–161.

Bolton, C., Gordon, D. and Turk, J.L. (1984b) Prostaglandin and thromboxane levels in central nervous system tissues from rats during the induction and development of experimental allergic encephalomyelitis (EAE). Immunopharmacology 7, 101–107.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cirino, G. and Flower, R.J. (1987) Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery in vitro. Prostaglandins 34, 59–62.

Cirino, G., Flower, R.J., Browning, J.L., Sinclair, L.K. and Pepinsky, R.B. (1987) Recombinant human lipocortin 1 inhibits thromboxane release from guinea pig isolated perfused lung. Nature 328, 270–272.

Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L. and Pepinsky, R.B. (1989) Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. Proc. Natl. Acad. Sci. U.S.A. 86, 3428–3432.

Colpaert, F.C., Meert, T., De Viltie, P. and Schmitt, P. (1982) Further evidence validating adjuvant arthritis as an experimental model of chronic pain in the rat. Life Sci. 31, 67–75.

Comera, C., Rothhut, B., Cavadore, J.C., Vilgrain, I., Cochet, C., Chambaz, E. and Russo-Marie, R. (1989) Further characterisation of four lipocortins from human peripheral blood mononuclear cells. J. Cell. Biochem. 40, 361–370.

Davies, P. (1985) Macrophages as effector cells. Fed. Proc. 44, 2925–2926.

De Castro Costa, M., De Sutter, P., Gybels, J. and van Hees, J. (1981) Adjuvant-induced arthritis in rats: a possible animal model of chronic pain. Pain 10, 173–185.

Errasfa, M. and Russo-Marie, F. (1989) A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. Br. J. Pharmacol. 97, 1051–1058.

Errasfa, M., Rothhut, B., Fradin, A., Biullardon, C., Junien, J.L., Bure, J. and Russo-Marie, F. (1985) The presence of lipocortin in human embryonic skin fibroblasts and its regulation by anti-inflammatory steroids. Biochim. Biophys. Acta 847, 247–254.

Flower, R.J. (1984) Macrocortin and the anti-phospholipase proteins. In: G. Weissmann (Ed.), Advances in Inflammation Research, Raven Press, New York, pp. 1–33.

Flower, R.J. (1988) Lipocortin and the mechanism of action of the glucocorticoids. Br. J. Pharmacol. 94, 987–1015.

Flower, R.J. and Blackwell, G.J. (1979) Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. Nature 278, 456–459.

Fu, S.C., Mozzi, R., Krakowka, S., Higgins, R.J. and Horrocks, L.A. (1980) Plasmalogenase and phospholipase A_1, A_2 and L_1 activities in white matter in canine distemper virus-associated demyelinating encephalomyelitis. Acta Neuropathol. 49, 13–18.

Geczy, C.L. (1984) The role of lymphokines in delayed-type hypersensitivity reactions. Springer Semin. Immunopathol. 7, 321–346.

Goulding, N.J., Godolphin, J.L., Sampson, M.B., Maddison, P.J. and Flower, R.J. (1989) Hydrocortisone induces lipocortin 1 production by peripheral blood mononuclear cells in vivo in man. Trans. Biochem. Soc. (in press).

Hirata, F., Schiffman, D., Venkatasubramanian, K., Salomon, D. and Axelrod, J.A. (1980) Phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc. Natl. Acad. Sci. U.S.A. 77, 2533–2536.

Hollenberg, M.D., Valentine-Braun, K.A. and Northup, J.K. (1988) Protein tyrosine kinase substrates: Rosetta Stones or simply structural elements? Trends Pharmacol. Sci. 9, 63–66.

Humes, J.L., Bonney, R.J., Pelus, L., Dahlgren, M.E., Sadowski, S.J., Kuehl, F.A. and Davies, P. (1977) Macrophages synthesise and release prostaglandins in response to inflammatory stimuli. Nature 269, 149–151.

Johnson, M.D., Kamso-Pratt, J.M., Whetsell, W.O. and Pepinsky, B. (1989a) Lipocortin-1 immunoreactivity in the normal human central nervous system and lesions with astrocytosis. Am. J. Clin. Pathol. 92, 424–429.

Johnson, M.D., Kamso-Pratt, J., Pepinsky, R.B. and Whetsell, W.O. (1989b) Lipocortin-1 immunoreactivity in central and peripheral nervous system glial tumours. Hum. Pathol. 20, 772–776.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

Lassmann, H. (1983) Chronic relapsing experimental allergic encephalomyelitis: its value as an experimental model for multiple sclerosis. J. Neurol. 229, 207–220.

Levine, S. and Strebel, R. (1969) Allergic encephalomyelitis: inhibition of cellular passive transfer by exogenous and endogenous steroids. Experientia 25, 189–191.

Levine, S., Strebel, R., Wenk, E.J. and Harman, P.J. (1962) Suppression of experimental allergic encephalomyelitis by stress. Proc. Soc. Exp. Biol. Med. 109, 294–298.

Lundgren, J.D., Hirata, F., Maron, Z., Logun, C., Steel, L., Kaliner, M. and Shelhamer, J. (1988) Dexamethasone inhibits respiratory glycoconjugate secretion from feline airways in vitro by the induction of lipocortin (lipomodulin) synthesis. Am. Rev. Respir. Dis. 37, 353–357.

Mackenzie, F.J., Leonard, J.P. and Cuzner, M.L. (1989) Changes in lymphocyte β-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat. J. Neuroimmunol. 23, 93–100.

MacPhee, I.A.M., Antoni, F.A. and Mason, D.W. (1989) Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J. Exp. Med. 169, 431–445.

Miele, L., Cordella-Miele, E., Facchiano, A. and Mukherjee,

A.B. (1988) Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin 1. Nature 335, 726–730.

Nathan, C.F., Murray, H.W. and Cohn, Z.A. (1982) The macrophage as an effector cell. New Engl. J. Med. 303, 622–626.

Parente, L. and Flower, R.J. (1985) Hydrocortisone and macrocortin inhibit the zymosan-induced release of lyso-PAF from rat peritoneal leucocytes. Life Sci. 36, 1225–1231.

Parente, L., Di Rosa, M., Flower, R.J., Ghiara, P., Meli, R., Persico, P., Salmon, J.A. and Wood, J.N. (1984) Relationships between the anti-phospholipase and anti-inflammatory effects of glucocorticoid-induced proteins. Eur. J. Pharmacol. 99, 233–239.

Paterson, P.Y. (1976) Experimental autoimmune (allergic) encephalomyelitis: induction, pathogenesis and suppression. In: P.A. Miescher and J.K. Mueller-Eberhard (Eds.), Textbook of Immunopathology, Grune and Stratton, New York, pp. 179–213.

Pearson, C.M. (1956) Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proc. Soc. Exp. Biol. Med. 91, 95–101.

Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Falbel, T., Ribolini, A., Garwin, J. and Wallner, B.P. (1986) Purification and partial sequence analysis of a 37KDa protein that inhibits phospholipase A_2 activity from rat peritoneal exudates. J. Biol. Chem. 261, 4239–4246.

Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Pingchang Chow, E., Burne, C., Huang, S.K., Pratt, D., Wachter, L., Hession, C., Frey, A.Z. and Wallner, B.P. (1988) Five distinct calcium and phospholipid binding proteins share homology with lipocortin 1. J. Biol. Chem. 263, 10799–10811.

Pepinsky, R.B., Sinclair, L.K., Chow, E.P. and O'Brine-Greco, B. (1989) A dimeric form of lipocortin-1 in human placenta. Biochem. J. 263, 97–103.

Piltch, A., Sun, L., Fava, R.A. and Hayashi, J. (1989) Lipocortin-independent effect of dexamethasone on phospholipase activity in a thymic epithelial cell line. Biochem. J. 261, 395–400.

Raine, C.S. (1984) Analysis of autoimmune demyelination: its impact upon multiple sclerosis. Lab. Invest. 50, 608–613.

Schauenstein, K., Fassler, R., Dietrich, H., Schwarz, S., Kromer, G. and Wick, G. (1987) Disturbed immune–endocrine communication in autoimmune disease. Lack of corticosterone response to immune signals in obese strain chickens with spontaneous autoimmune thyroiditis. J. Immunol. 139, 1830–1833.

Smith, M.E. (1979) Neutral protease activity in lymphocytes of Lewis rats with acute experimental allergic encephalomyelitis. Neurochem. Res. 23, 689–702.

Smith, M.E., Sedgewick, L.M. and Tagg, J.S. (1974) Proteolytic enzymes and experimental demyelination in the rat and monkey. J. Neurochem. 23, 965–971.

Smith, S.F., Goulding, N.J., Tetley, T.D., Godolphin, J.L., Guz, A. and Flower, R.J. (1989) A possible anti-inflammatory mechanism for glucocorticoids in the human lung. Clin. Sci. 76, 20P.

Traugott, U., Shevach, E., Chiba, J., Stone, S.H. and Raine, C.S. (1981) Autoimmune encephalomyelitis: simultaneous identification of T- and B-cells in target organ. Science 214, 1251–1252.

Traugott, U., Shevach, E., Chiba, J., Stone, S.H. and Raine, C.S. (1982) Acute experimental autoimmune encephalomyelitis: T- and B-cell distribution within the target organ. Cell. Immunol. 70, 345–356.

Trotter, J. and Smith, M.E. (1986) The role of phospholipases from inflammatory macrophages in demyelination. Neurochem. Res. 11, 349–361.

van Eden, W., Holoshitz, J., Nevo, Z., Frenkel, A., Klajman, A. and Cohen, I.R. (1985) Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. Proc. Natl. Acad. Sci. U.S.A. 82, 5117–5120.

Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Pingchang Chow, E., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature 320, 77–81.

Woelk, H. and Kanig, K. (1974) Phospholipid metabolism in experimental allergic encephalomyelitis: activity of brain phospholipase A_1 towards specifically labelled glycerophospholipids. J. Neurochem. 23, 745–750.

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Lipocortins (annexins) 1, 2, 4 and 5 are increased in the central nervous system in multiple sclerosis

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Summary

Western blotting and densitometry have been used to investigate the lipocortin content of post-mortem central nervous system (CNS) tissue samples from multiple sclerosis (MS) patients and normal controls. Lipocortins 1, 2, 4 and 5 were all detected in normal control grey and white matter. In white matter samples from MS patients these lipocortins were found to be significantly increased, a further elevation in lipocortin content was observed in MS plaque tissue. The implications of these findings with respect to the role of these proteins in inflammatory CNS disease and a possible mechanism of steroid action in the therapy of MS are discussed.

Introduction

MS is a chronic inflammatory and demyelinating disease of the CNS which may be immunologically mediated. The disease is characterized by the presence in the CNS of disseminated demyelinating lesions with perivascular infiltrates composed of macrophages, lymphocytes and plasma cells which may amalgamate to form plaques (Prineas and Wright, 1978). Studies have demonstrated the presence of oedema, intrathecal synthesis of IgG, abnormalities in T-cell subset ratios, and enhanced expression of MHC antigens and cytokines in the CNS of MS patients (Waksman, 1989). In addition, increased levels of pro-inflammatory enzymes such as phospholipase A_2 (PLA_2) (Woelk and Peiler-Ichikawa, 1974; Trotter and Smith, 1986) and PLA_2-derived ecosanoid mediators (Rosnowska et al., 1981; Bolton et al., 1984) have been observed in MS plaques and cerebrospinal fluid. Such findings are indicative of ongoing inflammatory and immune responses and may suggest dysregulation of these mechanisms.

Anti-inflammatory steroids have been shown to be beneficial in the treatment of early acute relapse of MS. Several studies have demonstrated that steroid therapy reduces the severity and duration of disease exacerbation (Goas et al., 1983;

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Compston et al., 1987). Although steroids reduce CNS oedema and modify various immunological abnormalities present in MS (Troiano et al., 1987) their precise mode of action is unknown. Conceivably their anti-inflammatory, anti-oedematous and immunomodulatory actions may all play a role in suppression of the disease.

It was proposed independently by several groups that the anti-inflammatory actions of the glucocorticoids are effected via the induction of mediator proteins which inhibit the activity of PLA_2 and were eventually named lipocortins (Di Rosa et al., 1984). At present six lipocortins have been identified (Pepinsky et al., 1988) and they are now recognised to be members of a larger family of structurally homologous, calcium- and phospholipid-binding proteins, termed the annexins (Crumpton and Dedman, 1990). With respect to their proposed function as glucocorticoid second messengers the most widely studied of these proteins is lipocortin 1. Many studies have demonstrated induction of lipocortin 1 following in vitro or in vivo treatment with glucocorticoids (Smillie et al., 1989; Goulding et al., 1990; Browning et al., 1990; Ambrose and Hunninghake, 1990; Solito et al., 1990), although it has not been demonstrable in all systems studied (Northup et al., 1988; Brönnegård et al., 1988) and the steroid inducibility of lipocortins 2 and 5 has yet to be established (Parente et al., 1990; Solito et al., 1990).

Inhibition of PLA_2 by lipocortins was observed in many early studies (Flower, 1988) and although the mechanism has become controversial (Davidson et al., 1987), lipocortins have been demonstrated to have anti-inflammatory actions both in vitro and in vivo. In vitro lipocortin 1 has been shown to inhibit release of ecosanoids from leukocytes, guinea-pig perfused lung and human umbilical artery preparations (Parente et al., 1984; Cirino et al., 1987; Cirino and Flower, 1987) and to reduce superoxide generation by activated phagocytes (Stevens et al., 1988; Maridonneau-Parini et al., 1989). In vivo lipocortin 1 suppresses some animal models of inflammation, including carrageenin-induced pleurisy and carrageenin- and PLA_2-induced paw oedema (Blackwell et al., 1982; Cirino et al., 1989). Lipocortin 1 also appears to have powerful effects in the CNS, such as inhibition of experimentally induced fever (Carey et al., 1990; Davidson et al., 1991) and prevention of ischaemic (Relton et al., 1991) and chemically induced (Black et al., 1991) neuronal damage. Recently recombinant human lipocortin 2 has also been shown to possess anti-phospholipase and anti-inflammatory activity (Parente et al., 1990).

There is much evidence to suggest that lipocortins have the ability to regulate powerful and potentially damaging inflammatory mechanisms. Thus these proteins may be endogenous regulatory molecules, and in inflammatory disease states such as MS steroid therapy may reduce inflammation by stimulating lipocortin production and thus down regulating PLA_2 activity. In this study we have investigated the distribution of lipocortins 1, 2, 4 and 5 in post-mortem CNS tissues from MS patients and control cases in an attempt to elucidate the potential role played by lipocortins in the regulation of inflammation in the CNS.

Materials and methods

CNS tissue samples

Coded samples of post-mortem brain and spinal cord from six control cases without neurological disease and seven cases of MS were obtained from the Multiple Sclerosis Society Tissue Bank at the Institute of Neurology, London. Clinical diagnosis of MS was confirmed by routine histological staining with haematoxylin, eosin and oil red O. The average age of the MS patients was 50 years (range, 33–74 years) and the duration of clinical disease 18 years (range 9 months–49 years). The mean interval between death and freezing was 41 h (range 27–82 h). Control cases had an average age of 38 years (range 20–50 years) and a mean death to freezing interval of 20 h (range 9–33 h). Medical records indicated that none of the patients had received steroid treatment within two years of death.

Small blocks of tissue were dissected from the MS cases to provide samples of: grey matter plaque without white matter, macroscopically normal-appearing white matter adjacent to plaques and apparently uninvolved white matter

remote from plaque areas. Four of the plaques exhibited perivenular inflammation, hypercellularity and macrophages containing oil red O-positive degenerating myelin and these were considered to be actively demyelinating lesions. Two hypocellular oil red O-negative demyelinated plaques were classified as chronic plaques. No positive oil red O staining or myelin loss was detected microscopically in the MS white matter samples. Control samples of white and grey matter were obtained by dissection from corresponding areas of brain and spinal cord of normal controls. In all, 35 samples of 100 mg each were stored at −70°C until use.

Preparation of tissues for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Each tissue sample was added to 0.5 ml of chilled homogenisation medium comprising 10 mM Hepes (*N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulphonic acid, sodium salt, Sigma), 5 mM EDTA (ethylenediamine tetraacetic acid, disodium salt, dihydrate, Sigma), 100 mM sodium chloride (Sigma) and 200 μM phenylmethylsulphonyl fluoride (Sigma) pH 7.6. Samples were chopped using scissors and then homogenised on ice by repeated passage through 19 and 21 gauge needles. The homogenates were then freeze/thawed twice and centrifuged at 15 000 × *g* for 30 min at 4°C. Supernatants were collected, assayed for total protein by the method of Bradford (1976) and diluted to 2 mg/ml protein with homogenisation medium. The diluted supernatants were then mixed in the ratio 1:1 with a sample buffer comprising 0.1 M Tris/Bicine (Tris(hydroxymethyl)aminoethane/*N*,*N*-bis[2-hydroxyethyl]glycine, Sigma), 2% sodium dodecyl sulphate (BDH), 5% 2-mercaptoethanol (Fisons), 10% sucrose (BDH) and 0.05% bromophenol blue (BDH), heated in a boiling water bath for 5 min and stored at −20°C prior to SDS-PAGE.

Detection of lipocortins by SDS-PAGE and immunoblotting

Constituent proteins were separated by SDS-PAGE essentially as described by Laemmli (1970) using a 'Mighty Small II' vertical slab gel unit (Hoefer Scientific Instruments, USA). Prepared supernatants containing 5, 10 or 20 μg protein were run on 10% acrylamide/0.26% bisacrylamide (BDH) resolving gels with 7.5% acrylamide/0.2% bisacrylamide stacking gels, for approximately 1 h at 35 mA/gel. Separated proteins were electrophoretically transferred to 0.45-μm nitrocellulose membranes (Schleicher & Schuell, Germany) by the method of Towbin et al. (1979) using a Transphor electrophoresis unit (Hoefer Scientific Instruments, USA) at 80 V for 1 h.

Lipocortins were visualised using a modified version of the methods of De Blas and Cherwinski (1983) and Frazer and Wisdom (1985). Following protein transfer the nitrocellulose membranes were incubated for 1 h with 3% milk powder in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-Tween) to block remaining protein binding sites. The membranes were then probed overnight in PBS-Tween with a 1:5000 dilution of rabbit antisera raised against one of the following: recombinant human lipocortin 1 (code no. 842), native human lipocortin 2 (code no. 774), native bovine lipocortin 4 (code no. 179) or native rat lipocortin 5 (code no. 890). All antisera were generously supplied by Dr. J.L. Browning, Biogen Research Corporation, USA. Cross-reactivity data for these antibodies are stated in Pepinsky et al. (1988). After washing twice for 15 min in PBS-Tween the membranes were incubated for 1 h with goat anti-rabbit IgG (Sigma) diluted 1:500 in PBS-Tween, then washed again and incubated for 1 h with a 1:10 000 dilution of rabbit peroxidase-anti-peroxidase complex (Sigma). Following two further washing steps first in PBS-Tween and then in PBS, the blots were incubated for 10 min with a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 0.01% ammonium nickel sulphate (Fisons) and 0.01% cobalt chloride (Sigma) in PBS. The membranes were then transferred to an identical solution containing in addition 0.01% H_2O_2, incubated until the colour was fully developed and then washed in distilled water. All incubations were performed at room temperature.

Densitometry of immunoblots

In order to provide a semi-quantitative assessment, the intensity of immunoblot bands was

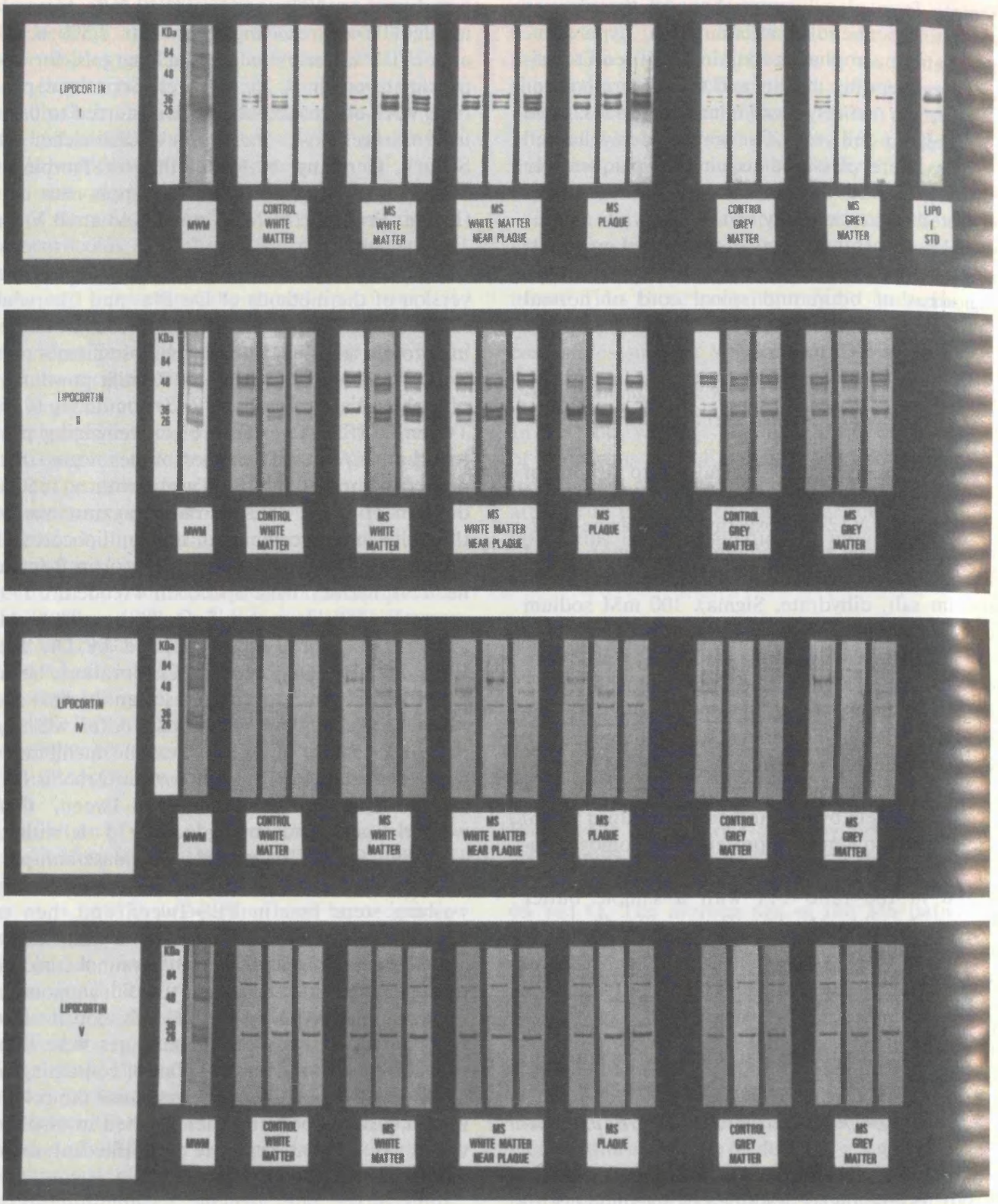

Fig. 1. Western blots of MS and control post-mortem CNS tissues probed for lipocortins 1, 2, 4 and 5. Supernatants from CNS homogenates were loaded at a total protein concentration per lane of: 5 μg for lipocortins 1 and 5, 10 μg for lipocortin 4 and 20 μg for lipocortin 2. Recombinant human lipocortin 1 standard was applied at 25 ng per lane. MWM, Sigma prestained molecular mass markers.

measured using a Joyce-Loebl Chromoscan 3. Results were expressed as integral values and following log transformation to stabilize the variance, were analysed by One Way Analysis of Variance. For comparison of white matter sub-types three orthogonal contrasts were fitted: control versus all MS white matter sub-types, remote MS white matter versus plaque and adjacent white matter, and adjacent white matter versus plaque.

Results

Representative Western blots of MS and control CNS tissues are shown in Fig. 1. In total 35 samples were assayed, with 5–7 samples per group. For clarity only three samples from each group are shown, although all 35 were quantified by densitometry. From Fig. 1 it can be seen that lipocortins 1, 2, 4 and 5 are present in both the grey and the white matter of normal control subjects. The molecular masses of immunoreactive proteins were estimated from a calibration curve constructed using Sigma prestained molecular mass markers. On blots probed for lipocortin 1 three distinct bands of 37, 35 and 33 kDa were consistently detected in almost all samples. The molecular mass of the 37 kDa band is concordant with that reported for lipocortin 1 (Pepinsky et al., 1988) and further confirmation of the identity of this band was provided by its co-migration with recombinant human lipocortin 1 standard. In addition to the presence in CNS samples of 35- and 33-kDa bands, a small amount of immunoreactive 33-kDa protein was also observed to be contaminating the lipocortin 1 standard. These lower molecular mass species have been observed by other workers and appear to be degradation products of lipocortin 1 (Huang et al., 1987; Smith et al., 1990a; Ambrose and Hunninghake, 1990). Following semiquantitation of the blots by densito-metry, a preliminary Two Way Analysis of Variance including these bands as a second factor showed there was no difference between any of the groups in the proportion of protein in each band (data not shown). Furthermore plots of protein level against death to freezing time for each group showed no indication of a relationship between protein concentration and death to freezing interval (data not shown).

Several bands between 30 and 37 kDa were observed on blots probed for lipocortin 2 and there was some cross-reactivity of this antibody with lipocortin 1, although the major band had a molecular mass of 35 kDa. The identity of this band as lipocortin 2 was later substantiated using a more specific antibody, code no. Ab 1, generously supplied by Dr. L. Parente, Sclavo, Italy

TABLE 1

DENSITOMETER VALUES FOR WESTERN BLOTS OF MS AND CONTROL CNS TISSUES PROBED FOR LIPOCORTINS 1, 2, 4 AND 5

The density of immunoblot bands was assessed using a Joyce-Loebl Chromoscan 3. Results are expressed as mean integral ± SD. For statistical analysis the data were log transformed to stabilize the variance and analysed by One Way Analysis of Variance. Three orthogonal contrasts were fitted for comparison of white matter sub-types

Tissue		*n*	Lipocortin 1		2		4		5	
White matter	Control	7	865 ± 247		981 ± 635		109 ± 57		915 ± 272	
	MS-remote from plaque	6	2551 ± 1387		1822 ± 1111		468 ± 399		1243 ± 184	
	MS-adjacent to plaque	5	2585 ± 1111	***	2771 ± 740 [c]	*** [a]	486 ± 168 [c]	**	1356 ± 428	*
	MS-plaque	6	2842 ± 1308		3086 ± 438		689 ± 548		1530 ± 703	
Grey matter	Control	5	830 ± 276		435 ± 318		78 ± 23		662 ± 318	
	MS	6	841 ± 353		612 ± 378		132 ± 44 [b]		707 ± 188	

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$ for all MS white matter sub-types versus control.

[a] $P < 0.02$ MS white matter remote from plaque versus plaque and adjacent white matter.

[b] $P < 0.05$ MS grey matter versus control grey matter.

n, number of samples per group (except [c], where $n = 4$).

(data not shown). For lipocortins 4 and 5 the main bands were 40 and 30 kDa, respectively. Unfortunately standards for lipocortins 2, 4 and 5 were not available; however, the principle bands observed after incubation with the relevant antibodies had very similar molecular masses to those reported by Pepinsky et al. (1988). On all blots bands of between 50 and 70 kDa were observed; these could be multimers of lipocortin (Ando et al., 1989; Pepinsky et al., 1989) or alternatively may be due to cross-reactivity of the antisera with a related protein, for example the 68-kDa lipocortin (Pepinsky et al., 1988).

An overall comparison of all MS white matter sub-types with control white matter shows that lipocortins 1, 2, 4 and 5 are significantly increased in MS tissue ($P < 0.001$, $P < 0.001$, $P < 0.01$, $P < 0.05$ respectively, Table 1). In addition to the increased amount of lipocortin observed in apparently normal MS white matter compared to controls, each lipocortin was further elevated in the white matter adjacent to a plaque and also in plaque tissue. Thus within the MS CNS, the tissue content of each lipocortin was: plaque > white matter adjacent to plaque > white matter remote from plaque. This pattern was observed for all four lipocortins although it reached statistical significance only for lipocortin 2. Within the plaque group there appeared to be no relationship between plaque subtype (i.e. active or chronic) and lipocortin content, although it should be noted that the sample numbers are small. Comparison of control and MS grey matter showed that there is a slight increase in all lipocortins in MS grey matter compared to controls, but this was only significant at $P < 0.05$ for lipocortin 4.

Discussion

In this study we have demonstrated that lipocortins 1, 2, 4 and 5 are present in both grey and white matter of normal control subjects. We have also shown that the levels of these lipocortins are consistently raised in white matter from MS patients and appear to be further increased in plaque tissue and adjacent white matter.

The detection of lipocortin 1 in human CNS tissue has been reported previously by Johnson et al. (1989a), although to our knowledge ours is the first study to demonstrate the occurrence of lipocortins 2, 4 and 5. Using immunohistochemistry, Johnson and coworkers have shown that in the normal human CNS lipocortin 1 immunoreactivity is localized primarily in ependymal cells and sub-ependymal astrocytes. Additionally, in studies on brain tissue from patients with various CNS diseases they have also observed staining for lipocortin 1 in reactive astrocytes and infiltrating macrophages (Johnson et al., 1989a,b). In preliminary immunohistochemical studies on MS plaque tissue we have found lipocortin 1 staining in astrocyte cell bodies and processes in both active and chronic plaques. In acute plaques lipocortin 1 immunoreactivity was also observed in monocytes/macrophages in the inflammatory infiltrate surrounding blood vessel walls (unpublished observations and Newcombe et al., 1991).

It has been proposed that lipocortins act as 'second messengers' of the anti-inflammatory effects of the glucocorticoids. Although none of the patients in this study had recently received steroid therapy, all are presumed to have possessed an intact and functional hypothalamic-pituitary-adrenal axis. It has been suggested by Munck and co-workers (Munck et al., 1984) that one of the main physiological functions of the circulating glucocorticoids is to regulate inflammatory and immune mechanisms. They propose that normal basal steroid levels have a constant modulatory effect on these systems and that when stress causes steroid levels to rise they serve to switch off inflammatory and immune mechanisms to prevent overshoot. Since lipocortins appear to be steroid-inducible molecules which possess anti-inflammatory and immunomodulatory effects (Flower, 1988; Hirata, 1989) it is conceivable that regulation of the immune system and inflammatory responses by endogenous glucocorticoids may be mediated via the lipocortins. Thus the presence of lipocortins in normal control CNS tissue may represent the basal level required for immune and inflammatory regulation. In support of this, recent work by Carey et al. (1990) and Relton et al. (1991) provides evidence for a physiological role for lipocortin 1 in the central actions

of the glucocorticoids and suggests that lipocortins may be endogenous regulators of damage in the brain.

Recently we have demonstrated the presence of lipocortins 1, 2 and 5 in the CNS of Lewis rats and shown that following the induction of an experimental counterpart of MS, namely experimental allergic encephalomyelitis, the levels of lipocortin 1 increase with the progression of clinical symptoms and then decrease during the recovery phase of the disease (Bolton et al., 1990). The amount of lipocortin in the CNS appears to correlate very closely with serum corticosterone levels (unpublished observation) which also rise during the disease and are thought to be responsible for the spontaneous recovery observed in this model (Levine et al., 1962; Bolton and Flower, 1989; Mackenzie et al., 1989; MacPhee et al., 1989). There is some evidence to suggest that stress may play a role in some MS exacerbations (Franklin et al., 1988; Grant et al., 1989) and that serum cortisol levels in MS are chronically or sporadically high (Reder et al., 1987). Thus the increased amount of lipocortin in MS tissue may be due to induction by endogenous steroids, possibly in combination with circulating or localized growth factors or cytokines (Browning et al., 1990). In support of the hypothesis that lipocortin 1 is glucocorticoid-regulated in man, Smith et al. (1990b) have recently demonstrated a correlation between the concentration of lipocortin 1 in bronchoalveolar lavage fluid (BALF) and serum cortisol levels.

If lipocortins do act as endogenous regulators of inflammation, the increased amount of lipocortin in the CNS of MS patients may represent an unsuccessful attempt to overcome the chronic inflammation present in this disease. Failure to control the inflammatory process may be due to inactivation of lipocortin. High concentrations of auto-antibodies to lipocortin 1 have been observed in the serum of patients with rheumatoid arthritis on high dose glucocorticoid therapy and it has been suggested that these antibodies may reduce the effectiveness of lipocortin (Goulding et al., 1989). In addition, studies on lipocortin 1 in BALF (Smith et al., 1990a) have shown that a greater proportion of lipocortin 1 is in a degraded form in patients with inflammatory lung disease and the authors suggest that enzymatic breakdown may cause inactivation of the protein, leading to uncontrolled inflammation. It should be noted, however, that we find no difference between MS and controls in the proportion of native lipocortin and lower molecular mass forms, which would suggest no difference in breakdown.

It is possible that in MS, for whatever reason, lipocortin levels are insufficient to suppress inflammation and the efficacy of steroid treatment may be due to induction of lipocortins which then rise to a level sufficient to alleviate symptoms of the disease. Several studies have demonstrated that therapeutic doses of glucocorticoids can cause induction of lipocortin 1 in human tissues. Goulding et al. (1990) have observed increased levels of lipocortin 1 in human blood monocytes and neutrophils following systemic administration of hydrocortisone and corticosteroid treatment has also been shown to increase lipocortin 1 in BALF from normal individuals and patients with inflammatory lung disease (Smith et al., 1989; Ambrose and Hunninghake, 1990).

In this study we have shown that lipocortins 1, 2, 4 and 5 are present in normal human CNS tissues and are increased in tissue samples from MS patients, particularly in plaque areas. The next step in understanding the function of these proteins in MS will be to further characterise the cell types with which the lipocortin is associated. It is hoped that such studies will lead to a better understanding of the role played by lipocortins in the physiological and pharmacological regulation of inflammatory CNS disease.

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References

Ambrose, M.P. and Hunninghake, G.W. (1990) Corticosteroids increase lipocortin 1 in BAL fluid from normal individuals and patients with lung disease. J. Appl. Physiol. 68, 1668–1671.

Ando, Y., Imamura, S., Owada, M.K., Kakunaga, T. and Kannagi, R. (1989) Cross-linking of lipocortin 1 and enhancement of its Ca^{2+} sensitivity by tissue transglutaminase. Biochem. Biophys. Res. Commun. 163, 944–951.

Black, M.D., Relton, J.K., Carey, F., Crossman, A.R. and Rothwell, N.J. (1991) Lipocortin-1 inhibits NMDA-induced neuronal damage. Br. J. Pharmacol. 104 (Suppl.), C34.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J., Langham, C.S.J., Parente, L., Persico, P., Russel-Smith, N.C. and Stone, D. (1982) Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. Br. J. Pharmacol. 76, 185–194.

Bolton, C. and Flower, R.J. (1989) The effects of the anti-glucocorticoid RU 38486 on steroid-mediated suppression of experimental allergic encephalomyelitis in the Lewis rat. Life Sci. 45, 97–104.

Bolton, C., Turner, A.M. and Turk, J.L. (1984) Prostaglandin levels in cerebrospinal fluid from multiple sclerosis patients in remmission and relapse. J. Neuroimmunol. 6, 151–159.

Bolton, C., Elderfield, A.-J. and Flower, R.J. (1990) The detection of lipocortins 1, 2 and 5 in central nervous system tissues from Lewis rats with acute experimental allergic encephalomyelitis. J. Neuroimmunol. 29, 173–181.

Brönnegård, M., Andersson, O., Edwall, D., Lund, J., Norstedt, G. and Carlstedt-Duke, J. (1988) Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol. Endocrinol. 88, 723–739.

Browning, J.L., Ward, M.P., Wallner, B.P. and Pepinsky, R.B. (1990) Studies on the structural properties of lipocortin-1 and the regulation of its synthesis by steroids. In: M. Melli and L. Parente (Eds.), Cytokines and Lipocortins in Inflammation and Differentiation. Wiley-Liss, New York, NY, pp. 27–45.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Carey, F., Forder, R., Edge, M.D., Greene, A.R., Horan, M.A., Strijbos, P.J.L.M. and Rothwell, N.J. (1990) Lipocortin 1 fragment modifies pyrogenic actions of cytokines in rats. Am. J. Physiol. 259, R266–R269.

Cirino, G. and Flower, R.J. (1987) Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery in vitro. Prostaglandins 34, 59–62.

Cirino, G., Flower, R.J., Browning, J.L., Sinclair, L.K. and Pepinsky, R.B. (1987) Recombinant human lipocortin 1 inhibits thromboxane release from guinea pig isolated perfused lung. Nature 328, 270–272.

Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L. and Pepinsky R.B. (1989) Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw oedema test. Proc. Natl. Acad. Sci. USA 86, 3428–3432.

Compston, D.A.S., Milligan, N.M. and Hughes, P.J. (1987) A double blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis. J. Neurol. Neurosurg. Psychiatr. 50, 517–522.

Crumpton, M.J. and Dedman, J.R. (1990) Protein terminology tangle. Nature 345, 212.

Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R. (1987) Inhibition of phospholipase A_2 by lipocortins and calpactins — an effect of binding to substrate phospholipids. J. Biol. Chem. 262, 1698–1705.

Davidson, J., Flower, R.J., Milton, A.S., Peers, S.H. and Rotondo, D. (1991) Antipyretic actions of human recombinant lipocortin-1. Br. J. Pharmacol. 102, 7–9.

De Blas, A.L. and Cherwinski, H.M. (1983) Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies. Anal. Biochem. 133, 214–219.

Di Rosa, M., Flower, R.J., Hirata, F., Parente, L. and Russo-Marie, F. (1984) Nomenclature announcement. Anti-phospholipase proteins. Prostaglandins 28, 441–442.

Flower, R.J. (1988) Lipocortin and the mechanism of action of the glucocorticoids. Br. J. Pharmacol. 79, 987–1015.

Franklin, G.M., Nelson, L.M., Heaton, R.K., Burks, J.S. and Thompson, D.S. (1988) Stress and its relationship to acute exacerbation in multiple sclerosis. Neurlogy 38 (Suppl. 1), 254.

Frazer, H.E. and Wisdom, G.B. (1985) Detection of auto-antigens by immunoblotting using a peroxidase-anti-peroxidase complex. J. Immunol. Method. 80, 221–225.

Grant, I., Brown, G.W., Harris, T., McDonald, W.I., Patterson, T. and Trimble, M.R. (1989) Severly threatening events and marked life difficulties preceding onset or exacerbation of multiple sclerosis. J. Neurol. Neurosurg. Psychiatr. 52, 8–13.

Goas, J.Y., Marion, J.L. and Missoum, A. (1983) High dose intravenous methylprednisolone in acute exacerbations of multiple sclerosis. J. Neurol. Neurosurg. Psychiatr. 46, 99.

Goulding, N.J., Podgorski, M.R., Hall, N.D., Flower, R.J., Browning, J.L., Pepinsky, R.B. and Maddison, P.J. (1989) Autoantibodies to recombinant lipocortin-1 in rheumatoid arthritis and systemic lupus erythematosus. Ann. Rheum. Dis. 48, 843–850.

Goulding, N.J., Godolphin, J.L., Sharland, P.R., Peers, S.H., Sampson, M., Maddison, P.J. and Flower, R.J. (1990) Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. Lancet 335, 1416–1418.

Hirata, F. (1989) The role of lipocortins in cellular function as a second messenger of glucocorticoids. In: R.P. Schleimer, N.H. Claman and A. Oronsky (Eds.), Anti-inflammatory Steroid Action. Basic and Clinical Aspects, Academic Press, London, pp. 67–95.

Huang, K.-S., McGray, P., Mattaliano, R.J., Burne, C., Chow, E.P., Sinclair, L.K. and Pepinsky, R.B. (1987) Purification and characterization of proteolytic fragments of lipocortin-

1 that inhibit phospholipase A_2. J. Biol. Chem. 262, 7639–7645.

Johnson, M.D., Kamso-Pratt, J.M., Whetsell, W.O. and Pepinsky, R.B. (1989a) Lipocortin-1 immunoreactivity in the normal human central nervous system and lesions with astrocytosis. Am. J. Clin. Pathol. 92, 424–429.

Johnson, M.D., Kamso-Pratt, J., Pepinsky, R.B. and Whetsell, W.O. (1989b) Lipocortin-1 immunoreactivity in central and peripheral nervous system glial tumours. Human Pathol. 20, 772–776.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

Levine, S., Strebel, R., Wenk, E.J. and Harman, P.J. (1962) Suppression of experimental allergic encephalomyelitis by stress. Proc. Soc. Exp. Biol. Med. 109, 294–298.

Mackenzie, F.J., Leonard, J.P. and Cuzner, M.L. (1989) Changes in lymphocyte β-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat. J. Neuroimmunol. 23, 93–100.

MacPhee, I.A.M., Antoni, F.A. and Mason, D.W. (1989) Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J. Exp. Med. 169, 431–445.

Maridonneau-Parini, J., Errasfa, M. and Russo-Marie, F. (1989) Inhibition of O_2^- generation by dexamethasone is mimicked by lipocortin 1 in alveolar macrophages. J. Clin. Invest. 83, 1936–1940.

Munck, A., Guyre, P.M. and Holbrook, N.J. (1984) Physiological functions of the glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. 5, 25–44.

Newcombe, J., Brar, A., Li, H. and Cuzner, M.L. (1991) Expression of the 14E antigen in glial cells in human normal and pathologic central nervous system tissues. In: Glial-Neuronal Interactions, Ann. N.Y. Acad. Sci. 633, 556–588.

Northup, J.K., Valentine-Braun, K.A., Johnson, L.K., Severson, D.L. and Hollenberg, M.D. (1988) Evaluation of the anti-inflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J. Clin. Invest. 82, 1347–1352.

Parente, L., Di Rosa, M., Flower R.J., Ghiara, P., Meli, R., Persico, P., Salmon, J.A. and Wood, J.N. (1984) Relationships between the anti-phospholipase and anti-inflammatory effects of glucocorticoid-induced proteins. Eur. J. Pharmacol. 99, 233–239.

Parente, L., Becherucci, C., Peretti, M., Solito, E., Mugridge, K.G., Galeotti, C.L., Raugei, G., Melli, M. and Sanso, M. (1990) Are the lipocortins the second messengers of the anti-inflammatory actions of the glucocorticoids? In: M. Melli and L. Parente (Eds.), Cytokines and Lipocortins in Inflammation and Differentiation, Wiley-Liss, New York, NY, pp. 55–68.

Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Chow, E.P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A.Z. and Wallner, B.P. (1988) Five distinct calcium and- phospholipid binding proteins share homology with lipocortin 1. J. Biol. Chem. 263, 10799–10811.

Pepinsky, R.B., Sinclair, L.K., Chow, E.P. and O'Brine-Greco, B. (1989) A dimeric form of lipocortin-1 in human placenta. Biochem. J. 263, 97–103.

Prineas, J. and Wright, R.G. (1978) Macrophages, lymphocytes and plasma cells in the perivascular compartment in chronic multiple sclerosis. Lab. Invest. 38, 409–421.

Reder, A.T., Lowy, M.T., Metlzer, H.Y. and Antel, J.P. (1987) Dexamethasone suppression test abnormalities in multiple sclerosis: Relation to ACTH therapy. Neurology 37, 849–853.

Relton, J.K., Strijbos, P.J.L.M., O'Shaughnessy, C.T., Carey, F., Forder, R.A., Tilders, F.J.H. and Rothwell, N.J. (1991) Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. J. Exp. Med. 174, 305–310.

Rosnowska, M., Cendrowski, W., Sobocinska, Z. and Wieczorkiewicz, A. (1981) Prostaglandins E_2 and $F_{2\alpha}$ in the cerebrospinal fluid in patients with multiple sclerosis. Acta Med. Pol. 22, 97–103.

Smillie, F., Peers, S.H., Elderfield, A.J., Bolton, C. and Flower, R.J. (1989) Differential regulation by glucocorticoids of intracellular lipocortin I, II and V in rat mixed peritoneal leukocytes. Br. J. Pharmacol. 97, 425P.

Smith, S.F., Goulding, N.J., Tetley, T.D., Godolphin, J.L., Guz, A. and Flower, R.J. (1989) A possible anti-inflammatory mechanism for glucocorticoids in the human lung. Clin. Sci. 76 (Suppl. 21), 20P.

Smith, S.F., Tetley, T.D., Guz, A. and Flower. R.J. (1990a) Detection of lipocortin 1 in human lung lavage fluid: Lipocortin degradation as a possible proteolytic mechanism in the control of inflammatory mediators and inflammation. Environ. Health Perspect. 85, 135–144.

Smith, S.F., Goulding, N.J., Godolphin, J.L., Tetley, T.D., Roberts, C.M., Guz, A. and Flower, R.J. (1990b) An assay for the assessment of lipocortin 1 levels in human lung lavage fluid. J. Immunol. Methods 131, 119–125.

Solito, E., Raugei, G., Melli, M. and Parente, L. (1990) Effect of dexamethasone and phorbol myristate acetate on lipocortin 1, 2 and 5 mRNA and protein synthesis. In: B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco and E. Gramstrom (Eds.), Advances in Prostaglandin, Thromboxane and Leukotriene Research, Vol 20, Raven Press, New York, NY, pp. 291–294.

Stevens, T.R.J., Drasdo, A.L., Peers, S.H., Hall, N.D. and Flower, R.J. (1988) Stimulus-specific inhibition of human neutrophil H_2O_2 production by human recombinant lipocortin 1. Br. J. Pharmacol. 93, 139P.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proceedure and some applications. Proc. Natl. Acad. Sci. USA 79, 4350–4354.

Troiano, R., Cook, S.D. and Dowling, P.C. (1987) Steroid therapy in multiple sclerosis. Arch. Neurol. 44, 803–807.

Trotter, J. and Smith, M.E. (1986) The role of phospholipases from inflammatory macrophages in demyelination. Neurochem. Res. 11, 349–361.

Waksman, B.H. (1989) Multiple sclerosis. Curr. Opin. Immunol. 1, 33–73.

Woelk, H. and Peiler-Ichikawa, K. (1974) On the activity of phospholipase A_2, compared with 1-Alk-1-enyl-2-acyl- and 1-alkyl-2-acyl-glycerophosphatides in multiple sclerosis. J. Neurol. 207, 319–326.

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Lipocortin 1 (annexin 1) immunoreactivity in the cervical spinal cord of Lewis rats with acute experimental allergic encephalomyelitis

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Summary

Spontaneous recovery from acute experimental allergic encephalomyelitis (EAE) by the Lewis rat is probably mediated by endogenous corticosteroids. It has been proposed that the anti-inflammatory actions of the glucocorticoids may be effected via the induction of mediator proteins termed lipocortins and recently we have demonstrated increased levels of lipocortin 1 in the central nervous system (CNS) of EAE-diseased rats (Bolton C., A-J. Elderfield and R.J. Flower (1990), J. Neuroimmunol. 29: 173–181). In this study, utilizing antisera raised against recombinant human lipocortin 1, immunohistochemistry and light microscopy have been used to determine the distribution of the protein in the cervical spinal cord of Lewis rats during EAE. In normal animals lipocortin 1 immunoreactivity was localized predominantly in the walls of larger blood vessels and to a lesser extent capillaries. The same staining pattern was found in adjuvant-inoculated controls. In sections from EAE-inoculated animals there was no change during the induction phase, but with the onset of clinical symptoms and the appearance of inflammatory infiltrates in the CNS, a marked increase in lipocortin 1 immunostaining was observed. This additional staining was due to widespread immunoreactivity of the lesions, was maximal at the height of disease and decreased following recovery and lesion regression. Within the lesions the vast majority of infiltrating lymphocytes and macrophages were positive for lipocortin 1, including some very heavily stained macrophage-like cells. Measurement of corticosterone in the sera of these animals showed that changes in lipocortin 1 immunostaining in the CNS during EAE closely parallel serum corticosterone levels.

Introduction

Acute exacerbations of multiple sclerosis (MS), an inflammatory and demyelinating disease of the CNS, are often treated with anti-inflammatory steroids (Troiano et al. 1987). Experimentally these compounds are also very effective in suppressing EAE, an animal model of MS (Rosenthale et al. 1969; Levine and Sowinski 1980; Bolton and Flower 1989). Furthermore there is considerable evidence that the spontaneous recovery which is characteristic of acute EAE in the Lewis rat is mediated by endogenous steroids released from the adrenal cortex. Several studies have demonstrated that serum corticosterone levels rise dramatically prior to the onset of remission (Levine et al. 1980; Mackenzie et al. 1989; MacPhee et al. 1989). If this glucocorticoid surge is prevented by adrenalectomy (Levine et al. 1962; MacPhee et al. 1989) or antagonized with the anti-glucocorticoid RU38486 (Bolton and Flower 1989) disease severity is increased and recovery delayed or abolished, effects which can be reversed by administration of exogenous steroids.

The mechanism of steroid action in MS and EAE has not been established. However, it has been proposed by several groups that the anti-inflammatory effects of the glucocorticoids are in part mediated by the induction of effector proteins termed lipocortins which may act by inhibiting the activity of phospholipase A_2 (PLA_2) and thus the generation of pro-inflammatory arachidonic acid metabolites (for review see Flower 1988). Lipocortins have recently been found to be members of the annexin family of calcium and phospholipid binding proteins (Crumpton and Dedman 1990). The most widely studied of these proteins, lipocortin 1, has been sequenced and the human gene

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cloned (Wallner et al. 1986). Several studies have demonstrated that the recombinant protein has anti-inflammatory properties in vivo (Miele et al. 1988; Cirino et al. 1989; Errasfa and Russo-Marie 1989). Recently, lipocortin 1 has been found to have biological activity in the CNS: when administered intravenously it can prevent febrile reactions in the rabbit (Davidson et al. 1991) and intracerebroventricular injection of lipocortin 1 inhibits cytokine induced fever in the rat (Carey et al. 1990). There is also emerging evidence that endogenous lipocortin 1 protects against neuronal damage in the brain. In a model of cerebral ischaemia, Relton and co-workers (1991) have observed increased expression of lipocortin 1 around infarcted areas, central injection of an N-terminal fragment of lipocortin 1 was found to reduce infarct size, conversely administration of a neutralizing antibody increased the extent of ischeamic injury and oedema. In addition lipocortin 1 fragment has also been shown to inhibit other types of neuronal damage, such as that mediated via the NMDA receptor (Black et al. 1991).

Previous studies by our group have demonstrated increased levels of lipocortins 1, 2, 4 and 5 in the CNS of MS patients (Elderfield et al. 1992) and elevated amounts of lipocortin 1 in the cerebellum and spinal cord of EAE-diseased rats (Bolton et al. 1990). In this study we have used immunohistochemistry to investigate the cellular distribution of lipocortin 1 in the cervical spinal cord of Lewis rats throughout the course of EAE. Identification of the cell types with which lipocortin 1 is associated in the target tissues of EAE, may provide further insight into the role of this protein in the regulation of inflammatory CNS disease.

Materials and methods

Induction of EAE

Male Lewis rats were obtained from Bantin and Kingman (Hull, UK) and allowed at least one week to adapt to their new environment prior to the start of an experiment. Animals were housed 4–6 per cage with free access to CRM rat diet and water. For the induction of EAE, rats weighing 200–250 g were inoculated in each hind foot pad with 0.1 ml of an emulsion comprising guinea-pig spinal cord, incomplete Freund's adjuvant (Difco) and sterile phosphate buffered saline (PBS) in the ratio 1:1:1 plus 10 mg/ml killed *Mycobacterium tuberculosis* (H_{37} RA, Difco). Normal control animals were not inoculated and complete Freund's adjuvant (CFA) controls received an emulsion in which the guinea-pig spinal cord was substituted with sterile PBS. EAE-immunized animals were weighed and examined daily for clinical signs of disease. Normal and CFA controls were subjected to the same handling procedure.

Serum and CNS tissue samples

In two separate experiments CNS tissue and serum samples were taken from groups of EAE-inoculated animals at various stages of the disease. Samples were collected during the pre-clinical phase, on days 4 and 7 post-inoculation (PI); during the disease phase, on the first day of exhibiting either weight loss, flaccid tail, hind limb weakness or paralysis; and during the recovery phase, on the first day of complete absence of clinical symptoms but only from animals which had previously shown definite clinical signs, i.e. complete or partial paralysis. CFA controls were sampled 15 days PI, corresponding with peak disease activity in EAE-inoculated animals. To limit fluctuations in circulating glucocorticoids due to circadian variation, all samples were taken in the morning when serum corticosterone levels are at their lowest (De Boer and Van der Gugten 1987).

For the collection of samples animals were initially rendered unconscious by carbon dioxide inhalation and 2 ml of blood was extracted by cardiac puncture. The rats were then asphyxiated with carbon dioxide, exsanguinated and the first 3 cm of spinal cord dissected out.

Immunohistochemistry

Cervical spinal cords were bisected down the midline and one half was fixed for 90 min in Carnoy's fluid (Fava et al. 1989), dehydrated in three changes of absolute alcohol and cleared in cedar wood oil (Fisons) prior to embedding in paraffin. Longitudinal horizontal sections, 6 μm thick, were cut at one standard depth and mounted onto 3-aminopropyltriethoxysilane (Sigma) coated slides. In each experiment immunohistochemistry was performed on CNS tissue samples from three representative animals in each group.

For immunohistochemistry the slides were de-paraffinized in xylene and taken through graded alcohols to water. Sections were then blocked for 30 min with 10% goat serum (Sigma) and probed overnight at 4°C with a 1:250 dilution of rabbit polyclonal antisera raised against recombinant human lipocortin 1 (code No. 842). Cross-reactivity data for this antibody have been reported by Pepinsky et al. (1988). After washing three times for 5 min slides were incubated for 30 min with goat anti-rabbit IgG (Sigma) diluted 1:50, then washed again and incubated for a further 30 min with a 1:1000 dilution of rabbit peroxidase-anti-peroxidase complex (Sigma). Following three more washes the sections were incubated with 0.05% 3-3′-diaminobenzidine tetrahydrochloride (Aldrich) plus 0.03% hydrogen peroxide (BDH) until fully stained, when the reaction was terminated by rinsing in distilled water. All incubations and washing steps were performed at room temperature in PBS unless otherwise stated.

Control sections from normal and EAE-diseased animals were incubated either with PBS in place of all antibody reagents to test for endogenous peroxidase, or with PBS or non-immune rabbit serum instead of 842 to determine specificity of staining. To further assess the specificity of immunostaining, in some experiments lipocortin 1 antisera was diluted to 1:250 in PBS and aliquots incubated with a range of concentrations of recombinant human lipocortin 1 (0.1–100 μg/ml), firstly for 1 h at 37°C and then overnight at 4°C. This pre-adsorbed antisera was then used on sections from normal and EAE animals in place of 842, after which they were processed as described above.

All sections were lightly counterstained with Tolui-

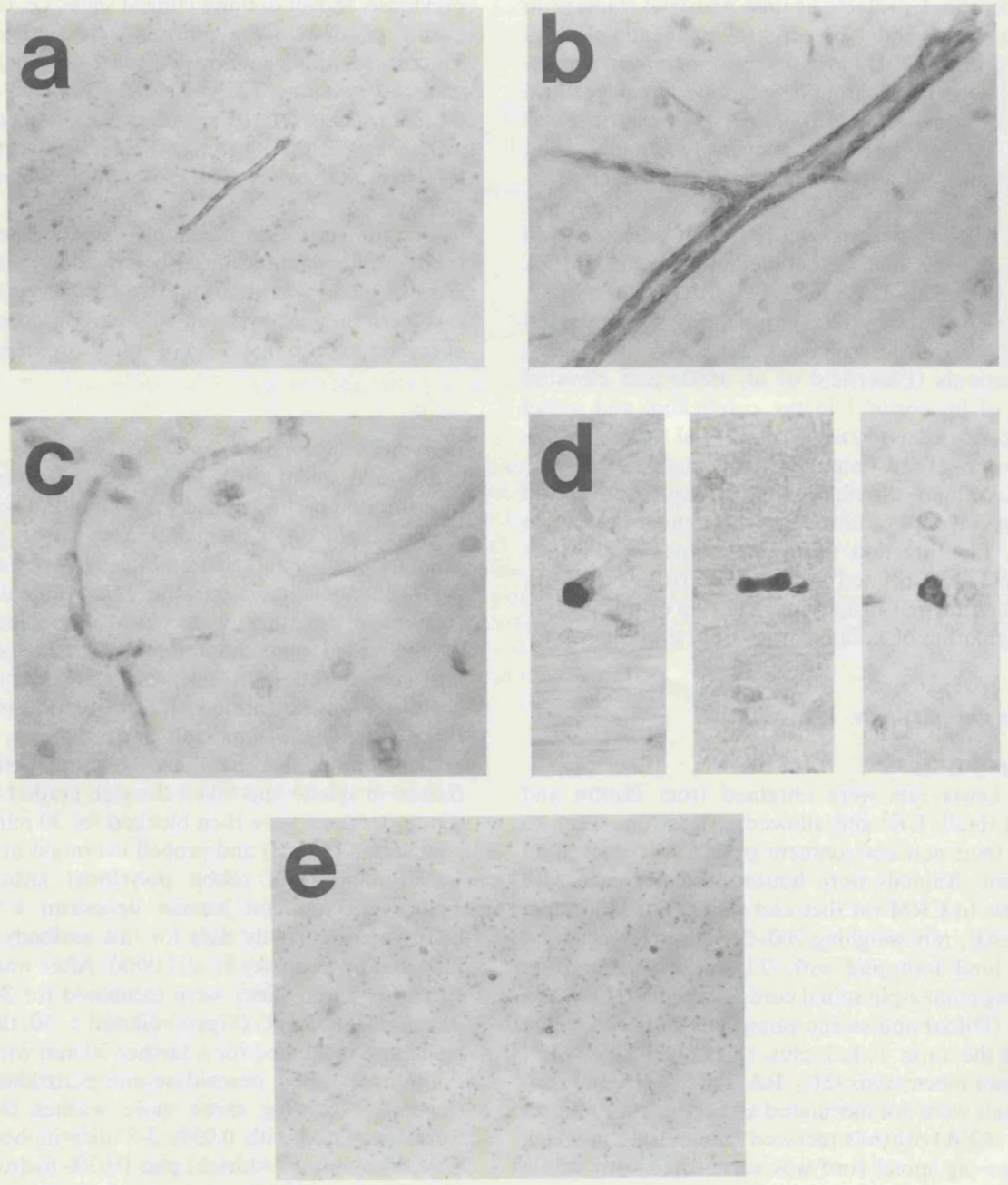

Fig. 1. Lipocortin 1 immunoreactivity in the cervical spinal cord of normal Lewis rats. Sections a–d were incubated with lipocortin 1 antisera. (a) Low power view (×100) showing localization of lipocortin 1 immunoreactivity in the walls of moderately stained blood vessels, plus very faint non-specific staining of neuronal nuclei. (b,c) Higher power fields of lipocortin 1 positive blood vessels (×400) and capillaries (×600) respectively. (d) Three typical examples of the very heavily stained cells which were occasionally observed within the lumen of blood vessels (×600). (e) Control section incubated with lipocortin 1 antisera pre-adsorbed with 100 μg/ml lipocortin 1 (×100).

dine Blue, dehydrated, cleared in xylene and mounted in DPX (BDH) prior to observation by light microscopy.

Corticosterone radioimmunoassay

Following cardiac puncture the blood was allowed to clot and serum samples were collected, clarified by centrifugation and stored at −20°C prior to assay. Serum corticosterone was measured in samples from all animals using an ICN RSL ^{125}I-Corticosterone Radioimmunoassay Kit (IDS, Tyne and Wear, UK) according to the manufacturer's instructions. Results were analysed using one-way ANOVA and the significance of differences between group means determined using the Newman-Keuls procedure (Snedecor and Cochran 1967).

Results

Lipocortin 1 immunohistochemistry

In cervical spinal cord sections from normal animals lipocortin 1 immunoreactivity was localized predominantly in the walls of larger blood vessels (Fig. 1A and B). However, staining was uneven and many blood vessels appeared unstained or only weakly positive. Some capillaries were also very faintly stained (Fig. 1C), although again most were below the visual limit of detection. Occasionally very heavily stained cells were observed located within the lumen of blood vessels and capillaries, as shown in Fig. 1D. No specific staining was associated with either neurones, astrocytes or oligodendrocytes. Control sections where lipocortin 1 antiserum was omitted or replaced with non-immune

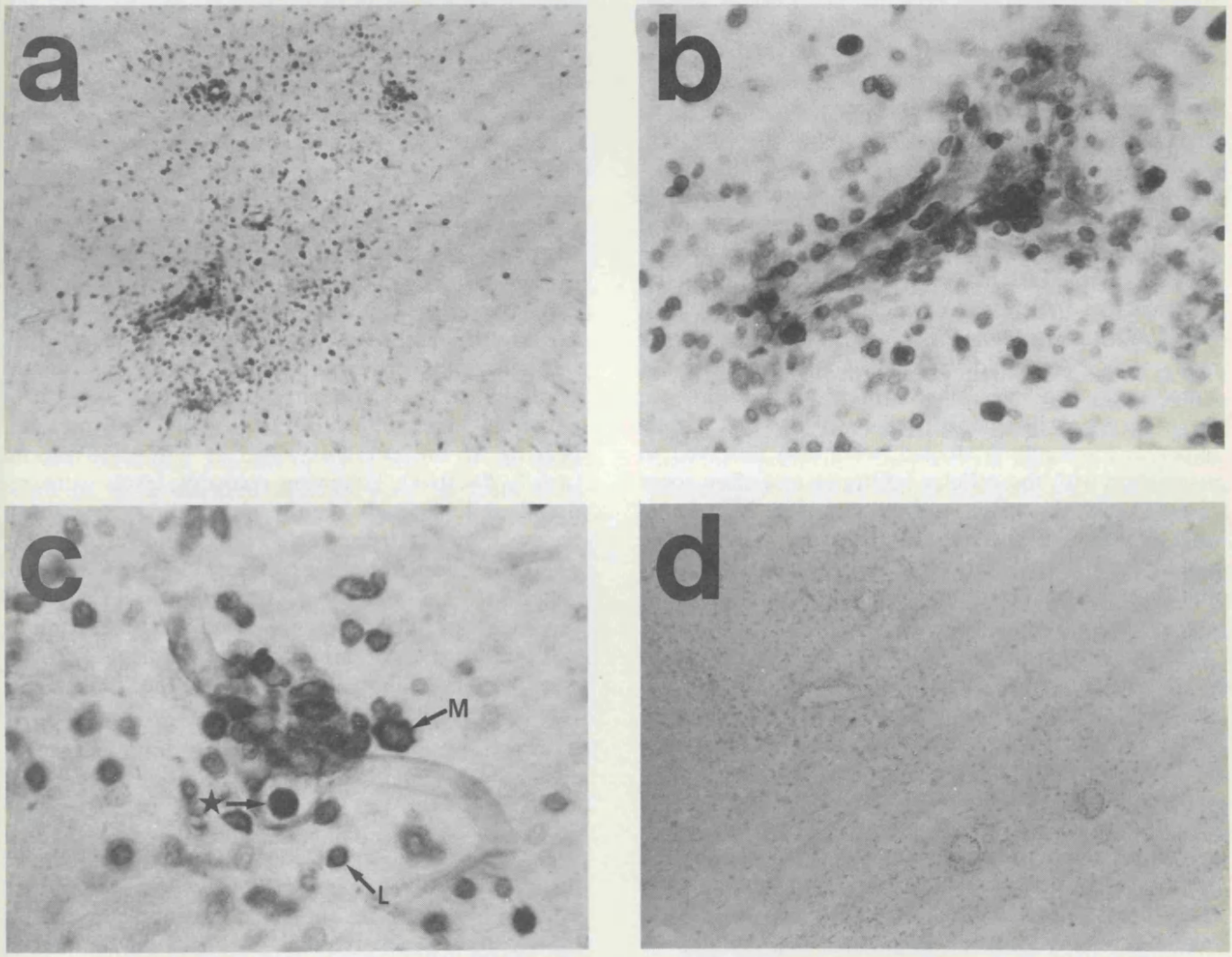

Fig. 2. Lipocortin 1 immunoreactivity in the cervical spinal cord of Lewis rats with EAE. All sections are from rats displaying symptoms of complete paralysis, a–c were incubated with lipocortin 1 antisera. (a) Low power view (×100) showing extensive immunostaining of several inflammatory lesions. (b) A typical perivascular lesion where the vast majority of infiltrating cells appear to be positive for lipocortin 1 (×400). (c) High power view (×600) of inflammatory cells adjacent to a small blood vessel illustrating lipocortin 1 immunostaining of a macrophage (M), a lymphocyte (L) and a very densely stained macrophage-like cell (*). (d) Control section incubated with antisera pre-adsorbed with 100 μg/ml lipocortin 1, several lesions can be seen lightly counterstained with toluidine blue, all immunostaining for lipocortin 1 was completely abolished (×100).

rabbit serum were completely negative. However, when sections were incubated with antisera, which had been pre-adsorbed with 100 mg/ml lipocortin 1, all vascular and intravascular staining was inhibited, although faint non-specific staining of neuronal nuclei was observed (Fig. 1E).

In the cervical spinal cord of EAE-inoculated animals, prior to the onset of clinical symptoms, the intensity and distribution of lipocortin 1 immunoreactivity followed the same pattern as that found in normal rats (not shown). However, in clinically sick animals the appearance of inflammatory lesions was associated with a marked increase in lipocortin 1 immunostaining (Fig. 2A). The extent of this staining appeared to be proportional to the number of lesions and the severity of disease: cords from rats exhibiting weight loss or flaccid tail, had only a few small lipocortin stained lesions, whereas progression of symptoms to hind limb weakness and paralysis was characterized by widespread staining of numerous infiltrates. Generally, fewer lipocortin-stained lesions were present in sections taken from animals which had recently recovered.

Staining of the lesions was quite variable: whilst a few were totally unstained, in many more all infiltrating cells appeared to be positive for lipocortin 1. In general, however, lipocortin 1 immunoreactivity was present in the vast majority of cells constituting the inflammatory infiltrates. A typical lesion surrounding a blood vessel is shown in Fig. 2B. Most of the cells were moderately stained and were morphologically typical of lymphocytes and macrophages (Fig. 2C). In addition, some very heavily stained cells, which were slightly larger and irregularly shaped, were also observed in association with the cellular infiltrates and often some distance into the surrounding tissue (Fig. 2C). These cells appeared to be similar to those occasionally seen within the blood vessels of normal animals (Fig. 1D) and their size and morphology suggests that these are also macrophage-type cells.

Throughout the course of EAE there appeared to be no difference in the type or proportion of cells positive for lipocortin 1, nor in the staining intensity of each cell-type. Furthermore staining of blood vessels did not change during the disease and neurones, oligodendroglia and astrocytes remained unstained. Sections from CFA controls were identical in intensity and pattern of immunoreactivity to those from normal and pre-diseased animals (not shown). On sections from EAE-diseased rats staining of lesions was completely inhibited when lipocortin 1 antisera was omitted or pre-adsorbed with 100 mg/ml recombinant lipocortin 1 (Fig. 2D).

Serum corticosterone

Corticosterone levels in the sera of rats in the experimental groups from which spinal cord samples were taken, are shown in Fig. 3. Baseline serum corticosterone in normal animals was 39 ± 51 ng/ml (mean $\pm$ SD, $n = 9$). In pre-diseased EAE-inoculated animals, corticosterone was slightly elevated at day 4 PI but had returned to baseline by day 7. However, clinically sick rats had serum corticosterone levels which were significantly higher than in normal controls and which progressively increased with the severity of symptoms, peaking at the paralysis stage at 276 ± 186 ng/ml ($n = 7$, $P < 0.01$). Following recovery, levels were reduced and although slightly elevated in comparison they were not statistically different to normal animals. The serum corticosterone concentration of CFA-inoculated rats was 138 ± 36 ng/ml ($n = 4$) and although this appeared to be higher than that of normal controls and somewhat lower than the value obtained for corresponding EAE-inoculated rats neither difference reached statistical significance.

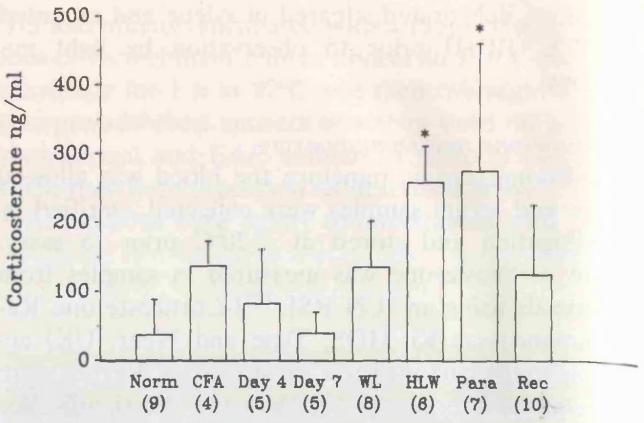

Fig. 3. Corticosterone levels in serum from normal and CFA-inoculated controls and from EAE-inoculated Lewis rats at various stages of the disease. Normal rats (Norm). CFA controls (CFA) were sampled 15 days PI. EAE-inoculated rats were sampled on day 4 or 7 PI or on the first day of exhibiting weight loss (WL), hind limb weakness (HLW), paralysis (Para) or recovery (Rec). Results are expressed as mean $\pm$ SD and are pooled from two separate experiments, the number of animals in each group is shown in parenthesis. Results were analysed using ANOVA and the significance of differences between group means determined using the Newman-Keuls procedure: * = $P < 0.01$ when compared to normal controls.

Discussion

In the cervical spinal cord of normal animals we found lipocortin 1 immunoreactivity localized in the walls of blood vessels and capillaries and recently we have observed a very similar distribution in human CNS tissue (Newcombe, Elderfield, Bolton and Flower, unpublished observation). Although Fava et al. (1989) were unable to detect lipocortin 1 in rat spinal cord, in studies on rat brain Strijbos and co-workers (1991) similarly observed patchy lipocortin 1 immunoreactivity in CNS blood vessels. In addition these workers found

brain tanycytes, ependymal cells and certain varicose nerve fibres and neuronal cell bodies were also positive for lipocortin 1. Interestingly immunoreactive lipocortin 1 was seen in the vascular tissue of all other organs studied by this group. The presence of lipocortin 1 immunoreactivity in capillaries as well as larger blood vessels suggests that the protein may be associated with the endothelium and in support of this several studies have demonstrated the occurrence of lipocortin 1 in cultured endothelial cells (Hullin et al. 1989; Fujimoto et al. 1990; Patte et al. 1991).

The emergence of clinical symptoms in EAE-inoculated rats was accompanied by a marked increase in lipocortin 1 immunostaining of the cervical spinal tissue, which was maximal at the height of disease and decreased following recovery, substantiating our previous findings using Western blotting (Bolton et al. 1990). Changes in the lipocortin content of the CNS occurred at the same time and followed the same pattern as serum corticosterone levels which rose dramatically with the development of symptoms. This surge in circulating glucocorticoids immediately prior to the onset of recovery is well documented (Levine et al. 1980; Mackenzie et al. 1989; MacPhee et al. 1989). Furthermore, the slight elevations in serum corticosterone apparent in EAE-inoculated rats during the induction phase and in CFA-immunized animals on day 15 PI have also been observed by other workers (Mackenzie et al. 1989) and probably reflect respectively, an acute reaction to the inoculum and the development of stress due to the induction of adjuvant arthritis (Bolton et al. 1990).

The increased amount of lipocortin 1 in the spinal cord of EAE-diseased animals resulted from widespread immunostaining of the inflammatory lesions which contained many lymphocytes and macrophages positive for lipocortin 1. In addition, some very heavily stained macrophage-like cells were observed in the vicinity of the infiltrates and the presence of similar cells within the lumen of blood vessels in normal animals suggests that these are blood derived. In support of these findings, an immunohistochemical study by Fava et al. (1989) also showed immunoreactive lipocortin 1 in intravascular and resident tissue macrophages in various rat organs and Johnson and co-workers (1989a,b) have found lipocortin 1 immunoreactivity in mononuclear cells infiltrating inflammatory CNS lesions in man. The association of lipocortin 1 with inflammatory cells is well established, the protein was originally isolated from rat peritoneal lavage fluid and macrophages are well documented as being a rich source of lipocortins (Flower 1988). Lower levels of lipocortin 1 and lipocortin 1 mRNA have also been detected in lymphocytes (Pepinsky et al. 1988; Brönnegård et al. 1988; Goulding et al. 1990a). Induction by steroids of lipocortin 1 in mononuclear cells has been demonstrated both in primary cultures (Browning et al. 1990; Goulding et al. 1990a) and in vivo in the rat and in humans (Solito et al. 1990; Goulding et al. 1990a), although other workers have found no induction (Brönnegård et al. 1988). In addition, Vishwanath et al. (1992) have recently shown that adrenalectomy causes a down-regulation of lipocortin 1 mRNA in rat tissues and that this can be prevented by administration of exogenous glucocorticoid, findings which lend weight to the concept that the gene for lipocortin 1 is under tonic steroid control. With respect to the present study it should be noted that although we have observed an apparent relationship between the lipocortin 1 content of the CNS and serum corticosterone levels this does not necessarily indicate steroid induction of the protein since our results could equally be due to an influx of lipocortin rich cells from the blood. Clarification of this point awaits further pharmacological studies at present underway in our laboratory.

EAE is principally a T-cell-mediated disease characterized histologically by the presence, primarily in the brain stem and spinal cord, of perivascular infiltrates composed mainly of lymphocytes, macrophages and plasma cells. Macrophages invading the CNS express MHC Class II antigens (Polman et al. 1986), appear to function in antigen presentation and also seem to be involved in demyelination (Lamper and Kies 1967), possibly via the action of PLA_2 (Trotter and Smith 1984). Increased levels of PLA_2-derived eicosanoid mediators have been found in the CNS of rats with EAE (Bolton et al. 1984). Mononuclear cells obviously play a pivotal role in EAE and several studies suggest that the lipocortin 1 they contain may influence many of the factors outlined above. Of particular relevance is the ability of the protein to suppress some in vivo models of inflammation (Miele et al. 1988; Cirino et al. 1989; Errasfa and Russo-Marie 1989). In addition, lipocortin 1 has been shown to prevent eicosanoid release from isolated tissues and leukocytes (Cirino and Flower 1987a,b; Cirino et al. 1987) which may be achieved by limiting the activity of PLA_2, although the mechanism of PLA_2 inhibition is controversial (Davidson et al. 1987). Early studies by Hirata and co-workers demonstrated that lipocortins possess immunomodulatory actions (Hirata 1989) and recently specific binding sites for lipocortin 1 have been discovered on the surface of monocytes (Goulding et al. 1990b). Clearly, there are many potential sites of action for lipocortin 1 in EAE.

The presence of lipocortin 1 in CNS endothelial cells may be of particular importance since increased blood brain barrier (BBB) permeability and CNS oedema are well defined features of EAE which are thought by many to be responsible for the physical symptoms of the disease (Levine et al. 1966; Simmons et al. 1982). The anti-oedematous properties of steroids

are well known and studies using computed tomography and magnetic resonance imaging suggest that corticosteroid therapy can reduce BBB abnormalities and oedema in the CNS of MS patients (Troiano et al. 1987; Kesselring et al. 1989). The location of lipocortin 1 in cells actively involved in the regulation of BBB function may indicate a role for this protein in the control of BBB permeability in EAE. Support for this suggestion is provided by the studies of Cirino et al. (1989) which have shown that local administration of recombinant human lipocortin 1 can mimic the prevention of peripheral oedema by steroids in the rat and Relton et al. (1991) who have recently provided evidence that lipocortin 1 may be an endogenous inhibitor of oedema in the brain.

The mechanism of steroid-induced recovery from EAE has not yet been established. One possible scenario which arises from the results presented in this paper is that by modulating the amount or activity of lipocortin 1, steroids may indirectly affect the function of endothelial cells, lymphocytes and macrophages and in this way influence the largely unknown chain of events leading to regression of oedema, migration of cells from the tissue and ultimately clinical recovery. It is hoped that elucidation of the role of lipocortins in steroid mediated self-cure, will provide further insight into the mechanism of steroid action in EAE and thus lead to improved therapies for MS and other steroid responsive diseases.

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References

Black, M.D., J.K. Relton, F. Carey, A.R. Crossman and N.J. Rothwell (1991) Lipocortin-1 inhibits NMDA-induced neuronal damage. Br. J. Pharmacol., 104 (suppl.): C34.

Bolton, C., A-J. Elderfield and R.J. Flower (1990) The detection of lipocortins 1, 2 and 5 in central nervous system tissues from Lewis rats with acute experimental allergic encephalomyelitis. J. Neuroimmunol., 29: 173–181.

Bolton, C. and R.J. Flower (1989) The effects of the anti-glucocorticoid RU38486 on steroid-mediated suppression of experimental allergic encephalomyelitis (EAE) in the Lewis rat. Life Sci., 45: 97–104.

Bolton, C., D. Gordon and J.L. Turk (1984) Prostaglandin and thromboxane levels in central nervous system tissues from rats during the induction and development of experimental allergic encephalomyelitis (EAE). Immunopharmacology, 7: 101–107.

Brönnegård, M., O. Andersson, D. Edwall, J. Lund, G. Norstedt and J. Carlstedt-Duke (1988) Human calpactin II (lipocortin 1) messenger ribonucleic acid is not induced by glucocorticoids. Mol. Endocrinol., 2: 732–739.

Browning, J.L., M.P. Ward, B.P. Wallner and R.B. Pepinsky (1990) Studies on the structural properties of lipocortin 1 and the regulation of its synthesis by steroids. In: M. Melli and L. Parente (Eds.), Cytokines and Lipocortins in Inflammation and Differentiation, Wiley-Liss, New York, pp. 27–45.

Carey, F., R. Forder, M.D. Edge, A.R. Greene, M.A. Horan, P.J.L.M. Strijbos and N.J. Rothwell (1990) Lipocortin 1 fragment modifies pyrogenic actions of cytokines in rats. Am. J. Physiol., 259: R266-R269.

Cirino, G. and R.J. Flower (1987a) The inhibitory effect of lipocortin on eicosanoid synthesis is dependent on Ca^{2+} ions. Br. J. Pharmacol., 92: 521P.

Cirino, G. and R.J. Flower (1987b) Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery in vitro. Prostaglandins, 34: 59–62.

Cirino, G., R.J. Flower, J.L. Browning, L.K. Sinclair and R.B. Pepinsky (1987) Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. Nature, 328: 270–272.

Cirino, G., S.H. Peers, R.J. Flower, J.L. Browning and R.B. Pepinsky (1989) Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. Proc. Natl. Acad. Sci. USA, 86: 3428–3432.

Crumpton, M.J. and J.R. Dedman (1990) Protein terminology tangle. Nature, 345: 212.

Davidson, F.F., E.A. Dennis, M. Powell and J.R. Glenney (1987) Inhibition of phospholipase A_2 by lipocortins and calpactins – an effect of binding to substrate phospholipids. J. Biol. Chem., 262: 1698–1705.

Davidson, J., R.J. Flower, A.S. Milton, S.H. Peers and D. Rotondo (1991) Antipyretic actions of human recombinant lipocortin-1. Br. J. Pharmacol., 102: 7–9.

De Boer, S.F. and J. Van der Gugten (1987) Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. Physiol. Behav., 40: 323–328.

Elderfield, A-J., J. Newcombe, C. Bolton and R.J. Flower (1992) Lipocortins (Annexins) 1, 2, 4 and 5 are increased in the central nervous system in multiple sclerosis. J. Neuroimmunol., 39: 91–100.

Errasfa, M. and F. Russo-Marie (1989) A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. Br. J. Pharmacol., 97: 1051–1058.

Fava, R.A., J. McKanna and S. Cohen (1989) Lipocortin 1 (p35) is abundant in a restricted number of differentiated cell types in adult organs. J. Cell. Physiol., 141: 284–293.

Flower, R.J. (1988) Lipocortin and the mechanism of action of the glucocorticoids. Br. J. Pharmacol., 94: 987–1015.

Fujimoto, M., T. Sakata, Y. Tsuruta, S. Iwagami, H. Teraoka, S-I. Mihara, Y. Fukiishi and M. Ide (1990) Enhancement of bradykinin-induced prostacyclin synthesis in porcine aortic endothelial cells by pertusis toxin. Possible implication of lipocortin 1. Biochem. Pharmacol., 40: 2661–2670.

Goulding, N.J., J.L. Godolphin, P.R. Sharland, S.H. Peers, M. Sampson, P.J. Maddison and R.J. Flower (1990a) Anti-inflammatory lipocortin 1 production by peripheral blood leukocytes in response to hydrocortisone. Lancet, 335: 1416–1418.

Goulding, N.J., P. Luying and P.M. Guyre (1990b) Characteristics of lipocortin 1 binding to the surface of human peripheral blood leucocytes. Biochem. Soc. Trans., 18: 1237–1238.

Hirata, F. (1989) The role of lipocortins in cellular function as a second messenger of glucocorticoids. In: R.P. Schleimer, N.H. Claman and A. Oronsky (Eds.), Anti-inflammatory Steroid Ac-

tion. Basic and Clinical Aspects, Academic Press, London, pp. 67–95.

Hullin, F., P. Raynal, J.M.F. Ragab-Thomas, J. Fauvel and H. Chap (1989) Effect of dexamethasone on prostaglandin synthesis and on lipocortin status in human endothelial cells. J. Biol. Chem., 264: 3506–3513.

Johnson, M.D., J. Kamso-Pratt, R.B. Pepinsky and W.O. Whetsell Jr. (1989a) Lipocortin-1 immunoreactivity in central and peripheral nervous system glial tumours. Hum. Pathol., 20: 772–776.

Johnson, M.D., J.M. Kamso-Pratt, W.O. Whetsell Jr. and R.B. Pepinsky (1989b) Lipocortin-1 immunoreactivity in the normal human central nervous system and lesions with astrocytosis. Am. J. Clin. Pathol., 92: 424–429.

Kesselring, J., D.H. Miller, D.G. MacManus, G. Johnson, N.M. Milligan, N. Scolding, D.A.S. Compston and W.I. McDonald (1989) Quantitative magnetic resonance imaging in multiple sclerosis: the effect of high dose intravenous methylprednisolone. J. Neurol. Neurosurg. Psychiatry, 52: 14–17.

Lamper, P.W. and M.W. Kies (1967) Mechanism of demyelination in allergic encephalomyelitis of guinea pigs. An electron microscopic study. Exp. Neurol., 18: 210–223.

Levine, S., J. Simon and E.J. Wenk (1966) Edema of the spinal cord in experimental allergic encephalomyelitis. Proc. Soc. Exp. Biol. Med., 123: 539–541.

Levine, S. and R. Sowinski (1980) Therapy of allergic encephalomyelitis in rats after onset of paralysis. In: A.N. Davison and M.L. Cuzner (Eds.), The Suppression of Experimental Allergic Encephalomyelitis and Multiple Sclerosis, Academic Press, London, pp. 199–209.

Levine, S., R. Sowinski and B. Steiner (1980) Effects of experimental allergic encephalomyelitis on thymus and adrenal in relation to remission and relapse. Proc. Soc. Exp. Biol. Med., 165: 218–224.

Levine, S., E.J. Wenk, T.N. Muldoon and S.G. Cohen (1962) Enhancement of experimental allergic encephalomyelitis by adrenalectomy. Proc. Soc. Exp. Biol. Med., 111: 383–385.

Mackenzie, F.J., J.P. Leonard and M.L. Cuzner (1989) Changes in lymphocyte β-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis. J. Neuroimmunol., 23: 93–100.

MacPhee, I.A.M., F.A. Antoni and D.W. Mason (1989) Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J. Exp. Med., 169: 431–445.

Miele, L., E. Cordella-Miele, A. Facchiano and A.B. Mukherjee (1988) Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin 1. Nature, 335: 726–730.

Patte, C., B. Rothhut, F. Russo-Marie and P.R. Blanquet (1991) Possible involvement of a lipocortin in the initiation of DNA synthesis by human endothelial cells. Exp. Cell Res., 197: 12–20.

Pepinsky, R.B., R. Tizard, R.J. Mattaliano, L.K. Sinclair, G.T. Miller, J.L. Browning, E.P. Chow, C. Burne, K-S. Huang, D. Pratt, L. Wachter, C. Hession, A.Z. Frey and B.P. Wallner (1988) Five distinct calcium and phospholipid binding proteins share homology with lipocortin 1. J. Biol. Chem., 263: 10799–10811.

Polman, C.H., C.D. Dijkstra, T. Sminia and J.C. Koetsier (1986) Immunohistological analysis of macrophages in the central nervous system of Lewis rats with acute experimental allergic encephalomyelitis. J. Neuroimmunol., 11: 215–222.

Relton, J.K., P.J.L.M. Strijbos, C.T. O'Shaughnessy, F. Carey, R.A. Forder, F.J.H. Tilders and N.J. Rothwell (1991) Lipocortin-1 is an endogenous inhibitor of ischaemic damage in the rat brain. J. Exp. Med., 174: 305–310.

Rosenthale, M.E., L.J. Datko, J. Kassarich and F. Schneider (1969) Chemotherapy of experimental allergic encephalomyelitis (EAE). Arch. Int. Pharmacod.T., 179: 251–275.

Simmons, R.D., C.C.A. Bernard, G. Singer and P.R. Carnegie (1982) Experimental allergic encephalomyelitis. An anatomically-based explanation of clinical progression in rodents. J. Neuroimmunol., 3: 307–318.

Snedecor, G.W. and W.G. Cochran (1967) Statistical Methods. 6th. ed. Iowa State University Press, Iowa.

Solito, E., G. Raugei, M. Melli and L. Parente (1990) Effect of dexamethasone and phorbol myristate acetate on lipocortin 1, 2 and 5 mRNA and protein synthesis. In: B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco and E. Gramstrom (Eds.), Advances in Prostaglandin, Thromboxane and Leukotriene Research, vol. 20, Raven Press, New York, pp. 291–294.

Strijbos, P.J.L.M., F.J.H. Tilders, F. Carey, R. Forder and N.J. Rothwell (1991) Localization of immunoreactive lipocortin-1 in the brain and pituitary gland of the rat. Effect of adrenalectomy, dexamethasone and colchicine treatment. Brain Res., 553: 249–260.

Troiano, R., S.D. Cook and P.C. Dowling (1987) Steroid therapy in multiple sclerosis. Arch.Neurol., 44: 803–807.

Trotter, J. and M.E. Smith (1984) Macrophage-mediated demyelination: The role of phospholipases and antibody. In: E.C. Alvord Jr., M.W. Kies and A.J. Suckling (Eds.), Experimental Allergic Encephalomyelitis: A Useful Model for Multiple Sclerosis, Alan R. Liss, New York, pp. 55–60.

Vishwanath, B.S., F.J. Frey, M. Bradbury, M.F. Dallman and B.M. Frey (1992) Adrenalectomy decreases lipocortin-1 messenger ribonucleic acid and tissue protein content in rats. Endocrinology, 130: 585–591.

Wallner, B.P., R.J. Mattaliano, C. Hession, R.L. Cate, R. Tizard, L.K. Sinclair, C. Foeller, E. Pingchang Chow, J.L. Browning, K.L. Ramachandran and R.B. Pepinsky (1986) Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature, 320: 77–81.